UNIVERSITY OF ALBERTA

HUMAN CYTOMEGALOVIRUS INDUCED VILLOUS TROPHOBLAST APOPTOSIS

By

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ABSTRACT

Congenital human cytomegalovirus (HCMV) infection can result in abortion or stillbirth with symptomatic survivors displaying sensorineural deficits and mental retardation. In addition to the neurological abnormalities, acquisition of primary maternal infection can often lead to neonates displaying intrauterine growth restriction (IUGR). HCMV infection results in inflammation to the villous placenta and the loss of villous trophoblast including the syncytiotrophoblast (ST) which acts as a barrier between maternal and fetal circulations and is responsible for nutrient exchange. Therefore, I hypothesize that infection by HCMV induces death to the villous trophoblast leading to the formation of lesions and impaired trophoblast function resulting in vertical transmission and IUGR neonates, respectively.

Initial experiments showed that HCMV infection of cultured trophoblast directly induced apoptosis and cell loss. Secretion of tumor necrosis factor (TNF)α in response to HCMV challenge was responsible for trophoblast death. Expression of immediate early (IE) proteins alone could also stimulate apoptosis. Interestingly, challenge with UV-inactivated HCMV resulted in apoptosis. Further investigation revealed binding of HCMV viral particles to toll-like receptor (TLR)2 resulted in secretion of TNFα.

A second model where HCMV infection indirectly leads to trophoblast death was also investigated. Intercellular cell adhesion molecule (ICAM)-1 expression is stimulated by HCMV in a paracrine fashion by TNFα and interleukin (IL)1β on non-infected cells. Expression in infected cells is induced in a manner independent of these two cytokines. HCMV IE proteins were also able to upregulate ICAM-1 expression. Consequently, monocytes, which bind to the ST surface via ICAM-1 / leukocyte function-associated

molecule (LFA)-1 interactions, showed increased adhesion to HCMV infected ST. Adherent monocytes were able to induce ST apoptosis via the release of TNFα.

I have demonstrated that HCMV can induce trophoblast death by a number of different mechanisms. The studies presented in this thesis have major implications to how HCMV infection induces inflammation of the villous placenta potentially leading to the formation of breaks in the ST barrier and deficiencies in trophoblast nutrient delivery to the fetus. Moreover, the data support the hypothesis that HCMV vertical transmission occurs through sites of damage and that HCMV infection impairs villous placenta function leading to IUGR pregnancies.

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ABBREVIATIONS

AIDS	Acquired immuno deficiency syndrome
ANOVA	Analysis of variance
AP-1	Activator protein-1
ATF	Activating transcription factor
Bad	Bcl-2/Bcl-XL associated death promoter
Bax	Bcl-2 associated protein
BCL	B-cell lymphoma/leukemia
BH3	BCL-2 homology domain 3
Bid	BH-3 interacting DD protein
BM	Basement membrane
cAMP	Cyclic adenosine monophosphate
c-fos	c-feline osteosarcoma
CHX	Cycloheximide
c-myc	c-myelcytomatosis oncogene
CT	Cytotrophoblast
CTL	Cytotoxic T lymphocyte
CREB	cAMP responsive element binding protein
DAPI	4,6-diamidino-2-phenylindole
DBs	Dense bodies
DD	Death domain
DED	Death effector domain
DIABLO	Direct IAP binding protein
DISC	Death-inducing signal complex
DMSO	Dimethyl Sulfoxide
E	Early
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGS	Ethylene glycol bis succinic acid N-hydrosuccinimide ester
ERK	Extracellular regulated kinase

ERV	Endogenous retrovirus
EVT	Extravillous trophoblast
FADD	Fas-associated death domain-containing protein
FBS	Fetal bovine serum
FITC	Fluorescein-5-isothiocyanate
g	Glycoprotein
GAPDH	Glyseraldehyde-3-phosphate dehydrogenase
gc	Glycoprotein complex
GFP	Green fluorescent protein
hCG	Human chorionic gonadotropin
HCMV	Human cytomegalovirus
HEL	Human embryonic lung
HLA	Human leukocyte antigens
HPL	Human placental lactogen
HUVEC	Human umbilical vein endothelial cells
IAP	Inhibitors of apoptosis
ICAM-1	Intercellular cell adhesion molecule-1
IE	Immediate early
IF	Infection focus
IFN	Interferon
Ig	Immunoglobulin
I-κB	Inhibitor of NF-κB
IKK	I-κB kinase
IL	Interleukin
IL-1R	Interleukin-1 receptor
IL-1RAcP	IL-1R accessory protein
IMDM	Iscove's Modified Dulbecco's medium
IRAK	IL-1R-associated kinase
IRF	Interferon regulatory factor
IUGR	Intrauterine growth restriction
JNK	c-Jun NH_2-terminal kinase

L	Late
LATs	Latency-associated transcripts
LFA-1	Leukocyte function-associated molecule-1
LIF	Leukemia inhibitory factor
LPS	Lipopolysacharide
MAC-1	C3 complement receptor
MCMV	Murine cyotmegalovirus
MEM	Minimum Eagle's medium
MHC	Major histocompatibility complex
MIE	Major immediate early
MMP	Matrix metalloproteinases
MOI	Multiplicity of infection
Mtd	Matador
MyD88	Myeloid differentiation factor 88
NF-κB	Nuclear factor κB
NK	Natural killer
p38	p38-mitogen associated kinase
p53	p53 tumor-suppressor protein
PAMPS	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEC	Placental endothelial cells
PLAP	Placenta alkaline phosphatase
RIP	Receptor-interacting protein
RT	Reverse transcriptase
RT-PCR	Reverse-transcriptase polymerase chain reaction
SCC	Standard saline citrate
SD	Standard deviation
Smac	Second mitochondria-derived activator of caspase
Sp1	Specificity protein 1
ST	Syncytiotrophoblast

CHAPTER 1: INTRODUCTION

1.1 Prologue

Human cytomegalovirus (HCMV), a member of the *Herpesviridae* family, causes a common infection found in the adult population and is one of the most common congenital infections in the world. Although HCMV infections are asymptomatic in normal healthy individuals, intrauterine transmission to the fetus during pregnancy can have severe consequences including death, mental retardation and sensorineural loss. Consequently, congenital HCMV infection has enormous public health impact and has been estimated by the Centers for Disease Control in the United States to cost nearly 2 billion US dollars each year to care for these infected children. The Institute of Medicine report, "Vaccinces for the 21st Century", recently gave the highest-level (Level 1) priority for HCMV vaccine development in the new millennium. A number of vaccines are in human clinical trials including a live attenuated vaccine, the Towne vaccine; however, there is large concern about its safety, particularly if inadvertently administered to a pregnant woman. Much of this concern is due to the limited knowledge about how HCMV interacts with placental cells and the mechanism of vertical transmission. It is widely believed that the vertical transmission occurs through the placenta and so a brief overview to the structure and function is presented. The later portion of the introduction will focus on HCMV, the interplay with the immune system and what is currently known about vertical transmission.

1.2 The Placenta

1.2.1 Structure and Function

The placenta is an organ that plays a crucial role in ensuring the proper development of the fetus during pregnancy. Not only is it responsible for attaching the fetus to the uterine wall but, because of its location between maternal and fetal tissue, it also performs a number of different functions including nutrient delivery, gas transfer, water balance, and elimination of metabolic waste (Benirschke and Kaufmann 2000). Furthermore, the placenta must act as an immunological barrier to prevent rejection by

maternal immune responses. Consequently, the limited presence of maternal leukocytes requires the placenta to also act as a barrier to maternal pathogens.

The mature human placenta is composed of the chorionic plate and the basal plate surrounding the intervillous space. The fetal vascularized villous trees project from the chorionic plate, which the umbilical cord supplying the fetal circulation is embedded, into the intervillous space and are bathed directly in the maternal blood that circulate through this region. Exchange of nutrients and waste between the maternal and fetal circulation takes place across the villous trees. The extensive system of villous trees is held in place through the anchoring villi which are attached to the basal plate.

1.2.2 Trophoblasts

During placental development two major structures, the anchoring and floating villi, are formed from three major types of trophoblast populations (Fig 1.1). These populations are derived from epithelial cytotrophoblast (CT) stem cells. CT found in the extravillous placenta differentiate into invasive CT where they invade the endometrium and parts of the myometrium thereby "anchoring" the placenta to the uterus. CT found in the floating villous placenta fuse into one continuous multinucleated cell layer called the syncytiotrophoblast (ST) which is in direct contact with maternal blood. Because the floating villi do not come in contact with the endometrium and thus "float" in the maternal blood, it is at this site that most of the maternofetal exchange takes place.

1.2.2.1 Extravillous Cytotrophoblast

Extravillous trophoblasts (EVT) are populations of trophoblast cells residing outside the villi. EVT display two phenotypes: the proliferative and invasive phenotype. Proliferative EVT are found exclusively in the basal lamina and represent immature EVT stem cells. These cells are characterized by the expression of proliferation marker Ki 67 (Bulmer *et al.* 1988), epidermal growth factor receptor (EGFR) c-erbB-1 (Jokhi *et al.* 1994) and epithelial integrin α6β4 (Damsky *et al.* 1992). The invasive phenotype EVTs are found deeper in the maternofetal interface and have irreversibly left the cell cycle. These differentiated trophoblast express c-erbB-2 (Jokhi *et al.* 1994), interstitial integrin α5β1(Damsky *et al.* 1992), and exhibit upregulated matrix metalloproteinases (MMP)-2

and 11 (Polette *et al.* 1994). Proliferating EVT are present during the first and second trimesters of pregnancy; however, by term EVT display considerably reduced proliferative activities (Benirschke and Kaufmann 2000).

1.2.2.2 Villous Cytophoblast

Incorporation of H-thymidine into villous CT but not ST and ultrastructural studies indicate CT are precursor stem cells responsible for maintaining the non-proliferating ST by fusing and releasing new cell material (Richart 1961; Boyd and Hamilton 1966). During early pregnancy this layer is nearly complete; however, by term this layer becomes discontinuous and can be found beneath only 20% of the ST (Benirschke and Kaufmann 2000). As pregnancy progresses, the amount of CT present does not decrease but rather as the surface area of the placenta increases the CT layer becomes separated (Simpson *et al.* 1992). This change is important since, unlike ST and endothelial cells, CT do not express Fc-γ receptors (Bright *et al.* 1994). Consequently, transport of immunoglobulins is only possible later in pregnancy when the CT layer becomes fragmented.

As CT differentiate, they exhibit higher levels of free ribosomes, rough endoplasmic reticulum and mitochondria (Burgos and Rodriguez 1966; Hamilton and Boyd 1966; Dempsey and Luse 1971; Jones and Fox 1977). Once the CT reaches an electron density similar to the overlaying ST, initial signs of fusion can be observed (Boyd and Hamilton 1966). Many hormones, growth factors and cytokines have been shown *in vitro* to regulate ST formation including simulating factors epidermal growth factor (EGF) (Morrish *et al.* 1987), human chorionic gonadotropin (hCG) (Cronier *et al.* 1994), cyclic adenosine monophosphate (cAMP) (Keryer *et al.* 1998) and estradiol (Cronier *et al.* 1999), and inhibiting factors transforming growth factor (TGF)-β1 (Morrish *et al.* 1991), and leukemia inhibitory factor (LIF) (Nachtigall *et al.* 1996). How these hormones regulate syncytial fusion remains unclear. It is likely that these factors regulate the expression of proteins such as CD98 (Kudo *et al.* 2003), connexin 43 (Cx43) (Frendo *et al.* 2003), cadherin-11 (Getsios and MacCalman 2003), syncytin (Blond *et al.* 1999) and endogenous retrovirus (ERV)-3 (Lin *et al.* 1999) all of which have been shown to be involved in trophoblast fusion.

1.2.2.3 Villous Syncytiotrophoblast

The ST is a single multinucleated cell layer that lines the villous placenta and thus separates maternal blood from fetal tissue. During earlier stages of pregnancy, the ST is a mostly homogeneous layer with evenly distributed cellular organelles and nuclei indicating no functional differentiation inside this layer, although recently fused regions are still distinguishable from the rest of the ST by the high density of organelles, high enzyme activity, and the unusual large nucleus size (Benirschke and Kaufmann 2000). However, as the placenta grows and the underlying CT layer becomes asymetric, the ST begins to undergo structural changes resulting in the formation of highly variable regions with specialized function yet still remaining a continuous cell layer without border (Burgos and Rodriguez 1966; Hamilton and Boyd 1966; Dempsey and Luse 1971; Jones and Fox 1977). From the 15th week on, regions with differing thinkness, composition of organelles and distribution of nuclei can be observed (Benirschke and Kaufmann 2000).

1.2.3 Trophoblast Turnover and Apoptosis

Apoptosis is mediated through two main pathways: the extrinsic (death receptor) pathway and the intrinsic (mitochondrial) pathway (Scaffidi *et al.* 1998). The extrinsic pathway is initiated by binding of death receptors to their cognate ligands, which results in a stepwise recruitment of adaptors and initiator caspases (particularly caspase 8) into the death-inducing signal complex (DISC). Initiation of the caspase cascade can directly lead to the induction of apoptosis or can trigger mitochondrial membrane permeablization, subsequently releasing pro-apoptotic factors such as cytochrome c (directly involved in the activation of caspases) and second mitochondria-derived activator of caspase (Smac)/direct IAP binding protein (DIABLO) [facilitate caspase activation by inhibiting proteins from the inhibitors of apoptosis (Iap) family], into the cytoplasm (Tsujimoto 2003). The intrinsic pathway is induced by various apoptotic stimuli that lead to mitochondrial permeablization and apoptosis. Mitochondrial membrane permeability is directly controlled by members of the B-cell lymphoma/leukemia (BCL)-2 family. The BCL-2 family of proteins can be divided into three subfamilies (Adams and Cory 1998): the anti-apoptotic subfamily (i.e. Bcl-2, Bcl-w and Bcl-x_L), the multi-domain pro-apoptotic subfamily [i.e. Bcl-2 associated protein

(Bax), Bcl-1 antagonist/killer (Bak) and matador (Mtd)] and the still growing BCL-2 homology domain 3 (BH3)-only protein subfamily [i.e. Bcl-2/Bcl-XL associated death promoter (Bad), BH-3 interacting DD protein (Bid) and Bcl-2-interacting mediator of cell death (Bim)] which seem to function as death signal sensors.

The turnover of villous trophoblast includes proliferation and differentiation of CT, fusion of underlying CT with ST, differentiation of ST, and the formation of syncytial knots that pinch off into the maternal circulation (Jones and Fox 1977). It now appears that apoptosis plays a major role in trophoblast turnover. Initiator capases such as caspase 8 are active in CT, whereas effector caspases such as caspase 3 are not (Huppertz *et al.* 1999). Structural signs of caspase 8 cleavage activity include cleavage of alpha-fodrin, a protein of the actin cytoskeleton, and externalization of phosphatidylserine (Huppertz *et al.* 1999; Huppertz *et al.* 1999). Activity of caspase 8 is crucial to syncytial fusion as inhibiting its activity by peptide inhibitor dramatically reduced fusion in villous explant cultures (Black *et al.* 2004). Activation of effector caspases in CT occurs rarely and only if overlying ST is present (Burton *et al.* 2003). The blocking of the progression of the apoptotic pathway may be in part due to the presence of anti-apoptotic members of the Bcl-2 protein family inside CT (Huppertz *et al.* 1998; Ho *et al.* 1999).

The progression of apoptosis does not occur immediately upon fusion of the CT with the ST (Huppertz *et al.* 1998). It is believed that the apoptotic cascade may be initially halted by the large amount of anti-apoptotic Bcl-2 family members present upon fusion (LeBrun *et al.* 1993; Sakuragi *et al.* 1994). The shift from inhibition of apoptosis to execution remains unclear; however, effector caspases 3 and 6 are eventually activated in the ST layer as demonstrated by caspase dependent cleavage of proteins such as CAD/ICAD, a DNase leading to the specific degradation of DNA (Huppertz *et al.* 2003), and cytokeratin 18, a major component of the cytoskeleton (Kadyrov *et al.* 2001). It should be emphasized that the activity of effector caspases are temporally and spatially regulated. Although the ST layer is a single multinucleated cell and does not have lateral membranes, DNA degradation and cytokeratin 18 cleavage are restricted to certain sites (Kadyrov *et al.* 2001; Huppertz *et al.* 2003). The last phase of apoptosis in the ST involve the accumulation of apoptotic nuclei into protrustions called syncytial knots.

These knots are eventually shed from the ST into the maternal circulation (Huppertz *et al.* 1998; Mayhew *et al.* 1999).

Villous trophoblast express TNFR p55 receptor (Yui *et al.* 1996) and are highly susceptible to the effects of the inflammatory cytokine tumor necrosis factor (TNF)α, a major inducer of apoptosis (Yui *et al.* 1994; Garcia-Lloret *et al.* 1996). Apoptosis stimulated by TNFα can be enhanced by interferon (IFN)γ in vitro (Yui *et al.* 1994), which alone, can not induce apoptosis. Although TNFα is found in normal healthy placental tissue and is produced by trophoblast (Li *et al.* 1992; Yang *et al.* 1993), suggesting a role in normal placental development (Monzon-Bordonaba *et al.* 2002), it is also clear that aberrant expression may lead to pathological placental conditions resulting in fetuses displaying intrauterine growth restriction (IUGR). Placentae isolated from rat and human pregnancies complicated with IUGR showed significant increases in the rate of apoptosis (Miller *et al.* 1996; Smith *et al.* 1997; Erel *et al.* 2001). Women with IUGR pregnancies had higher serum levels of TNFα (Bartha *et al.* 2003) and IUGR fetuses have elevated TNFα in their amniotic fluid (Heyborne *et al.* 1992). IUGR human placentae showed increased TNFα production (Holcberg *et al.* 2001) and increased susceptibility to TNFα (Crocker *et al.* 2003).

TNFα and IFNγ can also indirectly influence trophoblast apoptosis by stimulating surface intercellular cell adhesion molecule (ICAM)-1 expression, which mediates adhesion of maternal monocytes to the ST (Xiao *et al.* 1997). Adhering monocytes disrupts underlying ST culture by stimulating apoptosis in a TNFα-dependent manner (Garcia-Lloret *et al.* 2000).

1.2.4 Intrauterine Growth Restriction

IUGR is a failure of the fetus to achieve its full growth potential. IUGR fetuses have increased risk of fetal demise, and following birth an increased risk of cognitive dysfunction and cerebral palsy. As well, epidemiological studies have provided compelling evidence that IUGR predisposes individuals for coronary heart disease, hypertension, stroke and diabetes during adulthood (Barker *et al.* 1989; Barker *et al.* 1990). A significant proportion of IUGR cases are associated with abnormalities of the

placental structure and function, resulting in decreased oxygen and nutrient delivery by the placenta to the fetus (Robinson and Owens 1996).

Reduced trophoblast invasion leading to insufficient implantation is a key pathologic feature of IUGR pregnancies (Brosens *et al.* 1977). During the first half of pregnancy, uteroplacental arteries undergo a number of changes including replacement of endometrium and smooth muscle cells by invasive trophoblast and loss of elasticity and vasomotor control (Brosens *et al.* 1967). These changes result in the formation of wide incontractile tubes allowing for decreased maternal blood flow resistance and increased uterplacental perfusion. Subsequently, inadequate trophoblast invasion leads to reduced uteroplacental artery remodeling impairing oxygen and nutrient delivery to the placenta and fetus.

As previously mentioned, apoptosis is intertwined with the natural development of the human placenta, but elevated levels of apoptosis in the villous trophoblast have been demonstrated in IUGR (Smith *et al.* 1997; Erel *et al.* 2001; Ishihara *et al.* 2002). Villous tissue revealed enhanced p53 tumor-suppressor protein (p53) expression which can activate proapoptotic Bcl-2 family members such as Bax (Levy *et al.* 2002). Increased levels of TNFα were found in the maternal serum (Bartha *et al.* 2003) and amniotic fluid (Stallmach *et al.* 1995) from pregnancies complicated with IUGR. IUGR placentae exhibited elevated levels of TNFα production (Holcberg *et al.* 2001). In addition, cultured CT, ST and villous explants displayed increased susceptibility to the proapoptotic effects of TNFα (Crocker *et al.* 2003; Crocker *et al.* 2004). Taken together, these observations suggest apoptosis to be involved in the pathophysiologic mechanisms of IUGR.

1.3 Human Cytomegalovirus

1.3.1 Transmission and Epidemiology

HCMV is spread by close or intimate contact with another person who is shedding the virus, by vertical transmission from mother to fetus or by blood transfusion or organ transplantation from a HCMV-seropositive donor (Britt and Alford 1996). Infection with HCMV can be categorized into one of two groups: primary or recurrent. A primary infection is defined as the individual's first exposure to HCMV and is

characterized by prolonged excretion of the virus; whereas, recurrent infection is due to either reactivation of the individual's original strain of virus or a reinfection with a new strain of HCMV and is characterized by intermittent excretion of virus.

Humans are believed to be the only reservoir for HCMV. Seroepidemiologic studies have shown HCMV infection to be common world wide. The incidence of infection does not appear to be seasonal but prevalence is still influenced by a number of other major factors including age, cultural and socioeconomic status. In developed countries 40 to 60% of adults in the middle to upper socioeconomic status groups are seropositive for HCMV with this number rising to 80% in lower socioeconomic status groups (Demmler 1991). Almost the entire adult population in developing countries is HCMV positive.

1.3.2 Virus Structure

HCMV virion particles consist of three distinct regions: a 100 nm diameter icosahedral nucleocapsid containing a 230-kbp double stranded linear DNA genome surrounded by a layer defined as the tegument, which, in turn, is enclosed by a lipid bilayer derived from the host lipid membranes containing a number of viral glycoproteins.

1.3.2.1 Capsid Proteins

The capsid, the innermost structure, is an icosahedral structure comprised of at least five proteins: UL86 (the major capsid protein), UL85 (minor capsid protein), UL48-49 (the smallest capsid protein), UL46 (minor capsid binding protein), and UL80 (assembly protein). These proteins self-assemble into an icosahedral structure that incorporates unit length viral DNA.

1.3.2.2 Tegument Proteins

There have been at least 25 proteins that have identified in the tegument layer between the virion capsid and envelope. Tegument proteins appear have of number of different functions during HCMV replication including: the formation of the virion, intracellular particle transport, immune evasion, and stimulation of viral gene expression.

Because tegument proteins are present before viral gene transcription, it appears that tegument proteins may play an important role in insuring that the viral replication cycle can be efficiently initiated in the host cell. Accordingly, UL82 (pp71) enhances the infectivity of viral DNA and accelerates the HCMV replication cycle (Baldick *et al.* 1997) likely by transactivating immediate early viral promoters (Bresnahan and Shenk 2000; Schierling *et al.* 2004) and targeting cellular retinoblastoma family members for degradation (Kalejta *et al.* 2003; Kalejta and Shenk 2003). Furthermore, tegument protein UL83 (pp65) has been shown to block the induction of certain IFN-responsive genes (Browne and Shenk 2003; Abate *et al.* 2004).

1.3.2.3 Glycoproteins

HCMV expresses as many as 60 glycoproteins (g) which are embedded in a host-derived lipid bilayer. The functions of the majority of these envelope glycoprotein constituents remain unclear. Certain glycoproteins are known to form three major glycoprotein complexes (gc) on the virion membrane: gcI consisting primarily of gB; gcII thought to consist of gM and gN; and gcIII consisting primarily of gH and gL (Gretch *et al.* 1988).

HCMV gB, the major constituent of the viral envelope, plays a role in fusion of adjacent cells, cell-to-cell transmission, and targeting of progeny virus for release from the apical surface of polarized cells (Navarro *et al.* 1993; Tugizov *et al.* 1994; Tugizov *et al.* 1998; Jones *et al.* 2004). Polyclonal antisera raised against gB were capable of inhibiting viral infectivity (Britt and Mach 1996). Monoclonal gB-specific antibodies can inhibit virion binding (Ohizumi *et al.* 1992). Furthermore, soluble gB is able to neutralize HCMV gB-mediated fusion of virion envelope with cellular plasma membrane (Britt and Mach 1996; Boyle and Compton 1998). Taken together, evidence suggests that gB is involved in both attachment and fusion of the virion to the host cell membrane.

The second most abundant protein component of the virion envelope is gH. In a complex with gL, gH is thought to mediate viral/host cell membrane fusion. Anti-idiotypic antibodies which mimic the gH/gL complex were able to block virus penetration into the cell but not virion attachment (Keay and Baldwin 1991). Consistent with a role in membrane fusion, anti-gH antibody can inhibit cell-to-cell transmission of

HCMV (Rasmussen *et al.* 1984; Keay *et al.* 1989). The specific molecular mechanism employed by the gH/gL complex remains unknown.

A third glycoprotein highly expressed on the surface of the virion envelope is gM. HCMV gM forms a complex with gN; however, the function of this complex remains to be elucidated. Murine monoclonal antibodies against gN in the gM/gN complex can neutralize virus infectivity *in vitro* (> 98% plaque reduction) (Britt and Auger 1985). Interestingly, homologs found in alphaherpesviruses, pseudorabies virus and herpes simplex virus are dispensable for viral replication; whereas, deletion of gM from HCMV resulted in a null phenotype (Baines and Roizman 1991; Masse *et al.* 1999; Hobom *et al.* 2000).

1.3.3 Growth Cycle

1.3.3.1 Binding and Penetration

Initial attachment is mediated through a low affinity binding to cell surface heparan sulfate which is sensitive to competition by sulfated polymers such as heparin (Neyts *et al.* 1992; Compton *et al.* 1993). HCMV gcII appears to be the major envelope component responsible for the initial binding to heparin, and gB may also have minor contributions (Kari and Gehrz 1992; Ohizumi *et al.* 1992; Kari and Gehrz 1993; Boyle and Compton 1998). Following initial attachment to the host cell surface, the virus particle must fuse with the host membrane to release its contents into the cytoplasm. Penetration appears to be mediated by gcIII (via gH) and gB (Keay and Baldwin 1991; Ohizumi *et al.* 1992; Britt and Mach 1996; Boyle and Compton 1998). Identification of cellular receptors responsible for interacting with HCMV glycoproteins to mediate viral entry has been elusive and has only recently began to be unraveled.

Cell surface heparin sulfate proteoglycans function to tether the virus to the cell membrane and are absolutely required, although not sufficient alone, for HCMV infection. Following the initial low affinity heparin sensitive binding state, HCMV attachment is converted to non-dissociable state suggesting that HCMV absorption is a sequential process involving multiple cellular receptors (Boyle and Compton 1998). EGFR has been reported as a cellular receptor for HCMV (Wang *et al.* 2003). Wang *et al.* demonstrated interactions between EGFR and gB by displacing bound EGF with

soluble gB as well as by coimmunoprecipitation of gB and EGFR after crosslinking adsorbed virus. EGFR is expressed on many HCMV-permissive cells but it is not expressed on macrophages and neutrophils (Ewald *et al.* 2003), both of which are efficiently infected by HCMV. This suggests the existence of other cellular receptors for HCMV. Other then EGFR, gB has been shown to interact with β1-containing integrins through a disintegrin-like domain (Feire *et al.* 2004). Interactions with integrins were required for internalization of HCMV tegument proteins, thus likely act at the level of virus fusion.

Although a number of cellular receptors have been implicated in viral binding and penetration, HCMV entry likely involves even more receptors. As mentioned HCMV gB interacts with cellular EGFR and integrins to mediate fusion; however, gH has also been shown to play a major role in viral fusion (Keay and Baldwin 1991). Receptors to gH and other HCMV glycoproteins remain to be elucidated.

1.3.3.2 Gene Regulation

The replication of HCMV is prolonged when compared with other members of the herpes family of viruses. During a productive infection, the HCMV genome is expressed in three temporal phases that can be marked by the expression of immediate early (IE), early (E), and late (L) genes (Fig 1.2). HCMV genes are transcribed in the infected nucleus by RNA polymerase II and the associated basal transcription machinery, which is regulated by host-encoded transcription factors whose activity may be stimulated by viral transactivators (Fortunato and Spector 1999). Permissiveness for HCMV replication depends more on the efficiency of early viral gene expression and subsequent viral DNA replication rather than attachment and/or penetration (Landolfo *et al.* 2003).

1.3.3.3 Characteristics and Functions of the IE Protiens

Major immediate early (MIE) genes are under the control of major *ie1/ie2* promoter-enhancer region. An ~500 bp segment upstream from the TATA box of the ie1/ie2 genes contains repeat elements with binding sites to cellular transcription factors nuclear factor (NF)-κB, activator protein (AP)-1, specificity protein (Sp)1, and cAMP

responsive element binding protein (CREB)/activating transcription factor (ATF) (Boshart *et al.* 1985). Infection by HCMV rapidly induces the DNA-binding activities of these factors (Boldogh *et al.* 1993; Yurochko *et al.* 1995; Yurochko *et al.* 1997) and binding to corresponding sites is thought to contribute to the strong activity of the MIE enhancer. In fact, activation of the NF-κB pathway is required for the efficient transactivation of the MIE promoter (DeMeritt *et al.* 2004). The activity of the MIE enhancer is dependent of cell type and differentiation type (Meier and Stinski 1996). The availability of appropriate transcription factors in a specific cell type or during different states of differentiation is thought to be the reason for this specificity.

Three predominant IE gene products, IE1-72, IE2-86 and IE2-55, are derived from alternative splicing from a single major locus, *ie1/ie2*, located downstream of the MIEP. IE gene products regulate the transcription of various viral and cellular genes by considerably different mechanisms. IE1-72 can directly influence its own and cellular gene transcription by increasing levels of NF-κB activity via interaction with Sp1 (as well as other transcription factors) (Yurochko *et al.* 1995; Yurochko *et al.* 1997). IE2-86 is the predominant viral regulatory protein controlling the switch from IE to E and L gene expression during production infection (Fortunato and Spector 1999). Studies have revealed a physical association of IE2 with a number of cellular transcription factors including TATA-box-binding protein (TBP) and Sp1 (Caswell *et al.* 1993; Scully *et al.* 1995). Similar to IE1-72, cooperation with Sp1 stimulates NF-κB levels (Yurochko *et al.* 1995; Yurochko *et al.* 1997). IE1-72 and IE2-86 have been shown to work cooperatively for more efficient gene transactivation (Mocarski and Courcell 2001). The function of IE2-55, an alternatively spliced version of IE2-86, has also been shown to induce the levels of NF-κB by transactivating both the p105/p50 (Sp1-independent) and p65 (Sp1-dependent) promoters (Yurochko *et al.* 1995; Yurochko *et al.* 1997).

1.3.3.4 Latency and Reactivation

Following a primary infection, HCMV enters a state of latency where the viral genome is still present but gene expression is limited and infectious virus is not produced. It is thought that cells in the bone marrow and peripheral blood are the major sites of latency (Taylor-Wiedeman *et al.* 1991; Kondo *et al.* 1994), although studies have also

indicated that endothelial cells in arterial walls may also serve this function (Fish *et al.* 1998). HCMV DNA is found in peripheral blood monocytes and in CD34+ progenitor cells but neither is permissive for viral replication (Taylor-Wiedeman *et al.* 1991; Kondo *et al.* 1994).

The mechanism by which the viral genome is maintained in and reactivated from the latent state is poorly understood. DNA-binding factors multiprotein bridging factor (MBF)1 and Yin Yang (YY)1 bind to the IE promoter-enhancer region to repress IE gene expression in certain non-permissively infected cells (Shelbourn *et al.* 1989; Liu *et al.* 1994). Novel unspliced transcripts have been detected in granulocyte-macrophage progenitors that appear to be similar to latency-associated transcripts (LATs) found in cells latently infected with HSV (Kondo and Mocarski 1995). The role of these transcripts in latency remains to be determined. Activation of CD8 and CD4 T-cells and subsequent production of IFNγ and IL2 respectively leads to a specific macrophage phenotype capable of reactivating latent virus (Soderberg-Naucler *et al.* 2001).

1.3.4 Regulation of Apoptosis

Apoptosis is often used as a host defense mechanism against viruses to inhibit viral spread and persistence. Consequently, many viruses have developed mechanisms to inhibit the induction of apoptosis. Expression of HCMV IE1 and IE2 proteins alone is sufficient to inhibit TNFα induced but not UV light induced apoptosis (Zhu *et al.* 1995) suggesting these proteins act to inhibit receptor mediated apoptosis although the exact mechanism of inhibition is not apparent. HCMV E gene products can block receptor mediated signaling by relocalization of TNFRI from the cell surface to the perinucleus (Baillie *et al.* 2003). Further, HCMV UL36 gene encodes a cell-death suppressor, denoted viral inhibitor of caspase-8-induced apoptosis (vICA), which inhibits apoptosis by binding to the pro-domain of caspase 8 and preventing its activation (Skaletskaya *et al.* 2001). HCMV can also block apoptosis-associated with mitochondrial membrane permeabilization via the UL37 gene product, viral mitochondrial inhibitor of apoptosis (vMIA) (Goldmacher *et al.* 1999), by binding Bax and sequestering it at the mitochondria (Arnoult *et al.* 2004; Poncet *et al.* 2004). Virulent strains of HCMV, but not lab strain AD169, were found to encode a novel viral TNFR superfamily member from the UL144

open reading frame (Benedict *et al.* 1999). Unlike cellular TNFR, overexpression does not stimulate apoptosis and does not bind any known ligand of the TNF family implying that UL144 may contribute to the ability of HCMV to escape immune clearance. However, there is also no evidence that UL144 blocks apoptosis.

1.4 Human Cytomegalovirus and the Immune System

HCMV infection results in the induction of number of genes involved in cell cycle, apoptosis, interferon response and inflammatory response (Zhu *et al.* 1997; Zhu *et al.* 1998; Browne *et al.* 2001). UV-inactivated virus is able to upregulate a greater number of mRNAs than replication competent virus and many of the differently expressed mRNAs encode antiviral proteins (Browne and Shenk 2003). This discrepancy is likely due to HCMV encoding a number of viral proteins that function to inhibit antiviral responses. Nonetheless, fully infectious HCMV still triggers the transcription of a number mRNAs similar to UV-inactivated virus indicating that viral binding and penetration plays a role in regulating a number of host genes.

HCMV glycoproteins have been shown to alter cellular gene expression which is likely mediated by the rapid dysregulation of cellular transcription factors (Boldogh *et al.* 1993; Kowalik *et al.* 1993). Specific antibodies to gH and gB blocked the rapid activation of Sp1 and NF-κB by HCMV infection (Yurochko *et al.* 1997). Accordingly, soluble gB and anti-idiotypic antibody that mimics gH were able to induce Sp1 and NF-κB similarly to HCMV infection. Indeed, soluble gB can establish an antiviral state not dependent of IFN secretion (Boehme *et al.* 2004).

1.4.1 Toll-like Receptor Signaling

Toll-like receptors (TLRs) belong to an ancient family of receptor proteins involved in the innate immune response to various pathogens. TLRs are activated during infections by recognizing pathogen associated molecular patterns (PAMPs) commonly found on pathogens but not normally found on the host. Binding of PAMPs to TLRs stimulate signaling through the Toll/IL-1R (TIR) domain found in the cytoplasmic tail (Fig 1.3). Receptor activation triggers binding of the adaptor protein myeloid differentiation factor 88 (MyD88) to the TIR. IL-1R-associated kinase (IRAK) can then

bind and become phosphorylated which allows for activation of tumor necrosis factor receptor-associated factor 6 (TRAF6) resulting in the activation of NF-kB, c-Jun NH_2-terminal kinase (JNK), p38-mitogen associated kinase (p38) and extracellular regulated kinase (ERK) pathways. Activation of these pathways can then lead to the secretion of inflammatory cytokines including TNFα and IL-1.

To date 10 human TLRs have been identified. TLR3 and TLR4 have antiviral activity. TLR3 recognize dsRNA structure which is common to RNA virus replication while TLR4 has been implicated in recognition of respiratory syncytial virus (Alexopoulou *et al.* 2001; Haynes *et al.* 2001; Matsumoto *et al.* 2002). TLR3 and TLR4 also appear to work together to inhibit viral replication through an MyD88-independent pathway mediated by interferon regulatory factor (IRF)3 (Doyle *et al.* 2002; Doyle *et al.* 2003).

Binding of HCMV particles to cells led to the induction of inflammatory cytokines (Zhu *et al.* 1997). Compton *et al.* show that HCMV virions can activate an inflammatory response in a TLR2-dependent manner which could be enhanced by the presence of CD14 (Compton *et al.* 2003). Cells lacking either receptor were incapable of triggering an inflammatory response or activating the NF-κB pathway in response to HCMV. Soluble gB also induces secretion of inflammatory cytokines in a TLR2-dependent manner (Boehme and Compton 2004) and appears to directly interact with a heterodimer of TLR2 and TLR1 (Rassa and Ross 2003).

1.4.2 Interleukin-1 Signaling

The pro-inflammatory cytokine IL-1 plays a role in the regulation of many genes involved in immunity and inflammation, and has been implicated in the pathogenesis of several inflammatory diseases such as diabetes and rheumatoid arthritis (Mandrup-Poulsen *et al.* 1993; Arend and Dayer 1995). IL-1 is a family of cytokines to which receptor angonists IL-1α and IL-1β belong. Both proteins are synthesized as 31 kDa precursors that are proteolytically cleaved to generate mature 17 kDa forms and have comparable biological activities (Dower *et al.* 1986). A third member of the IL-1 family, IL-1ra, acts as a receptor antagonist by blocking binding and, consequently, the biological activites of IL-1α and IL-1β.

All three IL-1 isoforms can bind to two members of the interleukin-1 receptor (IL-1R) family, IL-1RI and IL-1RII although IL-RII preferentially binds IL-1β (Arend *et al.* 1994). The extracellular Ig-like domain structure of both receptors show high sequence homology; however, unlike IL-1RI, which signals though a 215 amino acid cytoplasmic tail containing a TIR domain, Il-1RII has only a 29 amino acid cytoplasmic domain which precludes signal transduction, thus functions as a decoy receptor (McMahan *et al.* 1991). Further, IL-1RII is shed from the cell surface preventing IL-1 from binding to IL-1RI and thus regulating extracellular IL-1 concentration.

IL-1RI requires IL-1R accessory protein (AcP) for signaling. Upon binding IL-1, IL-1RI and IL-1RAcP form a signaling complex which recruits MyD88 via the TIR domain (Fig 1.3). IRAK and IRAK-1 are then recruited followed by another adaptor, TRAF-6 which ultimately leads to the activation of IκB kinase (IKK) complex. IKK mediates phosphorylation of inhibitor-κB (IκB), releasing the NF-κB dimer allowing it to translocate to the nucleus.

HCMV is able to stimulate IL-1β expression via multiple mechanisms. First, binding of the viral particle alone to human monocytes can upregulate IL-1β mRNA resulting in increased production and secretion (Yurochko and Huang 1999). This induction can be mimicked with purified gB and anti-idiotypic antibody that mimics gH. Preincubation of virus with neutralizing antibody to gB and gH can abrogate induction. Second, HCMV IE proteins alone can transactivate IL-1β gene expression in a variety of cell lines (Iwamoto *et al.* 1990). HCMV IE2 proteins directly interact with the IL-1β promoter-bound Sp1 to upregulate IL-1β gene expression (Wara-Aswapati *et al.* 2003). Consequently, by inducing IL-1β HCMV is promoting its own replication since IL-1β can stimulate HCMV IE-promoter activity (Ritter *et al.* 2000).

1.4.3 Tumor Necrosis Factor α

TNFα plays a major role in the development of chronic inflammation and is produced by a wide variety of cells including macrophages and trophoblasts (Yang *et al.* 1993). Several lines of evidence indicate that TNFα contributes to tissue wasting that characterizes chronic inflammation. Transgenic mice bearing a TNFα transgene became severely wasted (Beutler 1993). Rabbits were found to lose nearly half their body weight

in response to macrophage-derived TNFα when infected with trypanosomes (Tracey *et al.* 1987). TNFα can induce intracellular adhesion molecules such as ICAM-1, and E-selectin on endothelial cells which can facilitate the recruitment of a number of cells in a chronic inflammatory response.

TNFα is expressed in two forms: a 26-kDa transmembrane protein found on the plasma membrane which is proteolytically cleaved by metalloproteinase to release a mature 17-kDa secreted form. Both forms of TNFα are present as trimers, and it is the trimeric structure that is important for its biological activity (Bazzoni and Beutler 1995). The biosynthesis of TNFα is highly regulated and can be induced by various pathogens including viruses, bacteria and parasites in macrophages. Induction of TNFα in macrophages during infection with *Mycobacteria tuberculosis* requires TLR2 activation (Underhill *et al.* 1999).

The multiple activities of TNFα are modulated through two distinct TNFRs, type 1 (p55 or p60) and type 2 (p75 or p80). These two receptors are found on virtually all cell types and bind to TNFα with high affinity but the exact role of both receptors in mediating the effects of TNFα is still debated. It is generally believed that TNFR1 is responsible for the majority of the biological activity of TNFα since it contains an intracellular death domain and TNFR2 does not. TNFR2 appears to preferentially bind the cell-associated form of TNFα (Lucas *et al.* 1997) but may also help to facilitate the interaction between TNFα and TNFR1 (Tartaglia *et al.* 1993). However, TNFR2 has been shown to mediate apoptosis independent of TNFR1 (Haridas *et al.* 1998).

TNFα induces association of TNFR1's death domains resulting in trimerization of the receptor (Gruss and Dower 1995). Subsequently, the adaptor molecule TNFR associated death domain (TRADD) binds to the clustered death domains via its own death domain (DD) (Fig 1.4). TRADD can then recruit a variety of signaling molecules to the activated receptor including TRAF2, receptor-interacting protein (RIP) and Fas associated death domain (FADD) (Hsu *et al.* 1996). Binding of FADD mediates activation of apoptosis through the caspase cascade. The death effector domain (DED) of FADD interacts with the DED of pro-caspase 8, which upon oligomerization activates itself through self cleavage to activate downstream executor pro-caspases such as caspase 3 resulting in apoptosis. Association of TRAF2 and RIP to TRADD opposes the activity

of FADD. TRAFF2 and RIP activate NF-κB inducing kinase (NIK), which in turn activates IKK leading to I-κB degradation and thus tranlocation of NF-κB to the nucleus. Further, TRAF2 and RIP can also stimulate the JNK/AP-1 pathway. Activation of NF-κB and JNK result in transcription of genes involved in cell survival and inflammatory responses (Dempsey *et al.* 2003). In most cells the anti-apoptotic signals induced by TNFα tend to outweigh the pro-apoptotic signals; thus, TNFR1 only signals for cell death in certain circumstances (e.g. when protein synthesis is blocked).

The effects of TNFα on HCMV replication appears to be dependent on the degree of differentiation of the infected cell. Addition of TNFα to differentiated HL-60 and mature THP-1 monocytic cell lines causes inhibition of IE enhancer/promoter activity (Stein *et al.* 1993). In contrast, TNFα stimulates HCMV IE enhancer/promoter activity in undifferentiated HL-60. Similar effects were seen in the murine macrophage cell line RAW 264.7 and the primary human umbilical vein endothelial cells (HUVEC) (Ritter *et al.* 2000; Lee *et al.* 2004). Activation of NF-κB by TNFα is responsible for stimulating the HCMV enhancer/promoter in immature HL-60 (Prosch *et al.* 1995). Interestingly, HCMV IE gene products can induce transcription factors necessary for TNFα promoter activity to further stimulate steady state mRNA and protein production (Geist *et al.* 1994; Geist *et al.* 1997).

1.4.4 Inhibition of Interferon Signaling

Interferons are a large family of secreted proteins involved in antiviral defense, cell growth regulations and immune activation. IFNs are commonly grouped into two distinct types. Type I IFNs, which include IFNα and IFNβ, are produced by most cell types in direct response to virus infection. Type II IFN, also known as IFNγ, is produced by cells of the immune system such as natural killer cells and activated T lymphocytes in response to virally infected cells. Both types of IFN stimulate an 'antiviral' state in target cells, whereby the replication of the virus is blocked by the synthesis of a number of enzymes that interfere with replication, slowing the growth of target cells and increasing susceptibility to apoptosis. The effectiveness of the IFN response had led many viruses, including HCMV, to develop mechanisms to counteract the actions of IFNs.

Infection of fibroblasts by UV-inactivated virus resulted in the upregulation of many more host genes than did replicative competent HCMV (Browne *et al.* 2001). Many of these genes, including those encoding interferon-responsive proteins and proinflammatory cytokines, are involved in host antiviral responses. This observation suggests that a number of viral gene products are responsible for the inhibition of antiviral pathways. HCMV blocks multiple steps in the IFNα signaling pathway thus blocking induction of IFNα-stimulated genes (Miller *et al.* 1999). In these studies inhibition of the IFNα pathway was carried out 48 hours post infection indicating viral gene products were necessary. However, IFN responsive genes can be upregulated within 6 hours of infection (Browne *et al.* 2001). High-density cDNA microarrays showed that cells infected with HCMV or treated with gB resulted in a transcription profile that did not differ significantly from IFN-treated cells (Simmen *et al.* 2001). De-novo protein synthesis was not required by HCMV-infected or gB-treated cells to elicit an interferon response demonstrating that induction of IFN-responsive genes were not likely mediated by secretion of IFNs or other small signaling molecules (Zhu *et al.* 1997; Boyle *et al.* 1999). In agreement, gB was able to establish an antiviral state in IFNα/β null cells (Boehme *et al.* 2004).

1.4.5 Monocytes/Macrophages

Monocytes are critical cells of the innate immune system responsible for protecting the body from infection though their release of soluble factors or cytokines and phagocytotic activity. Granulocyte-monocyte progenitor cells differentiate into promonocytes during hematopoiesis in the bone marrow. Promonocytes leave the bone marrow and enter the blood where they differentiate into mature monocytes. Monocytes circulate in the blood stream for about 8 hours after which they migrate into tissue and differentiate into resident tissue macrophages. Macrophages are five- to tenfold larger, contain a larger number of more complex intracellular organelles, have increased phagocytic ability, have higher levels of hydrolytic enzymes and secrete a larger variety of soluble factors than monocytes (Pike 1997).

There is evidence suggesting that monocytes serve as the vehicle for disseminating HCMV infection throughout the body. First, *in vivo* monocytes are the

primary target infected in blood during acute HCMV infection (Rice *et al.* 1984; Dudding *et al.* 1989; Fish *et al.* 1995) causing aberrant gene expression and immune response (Yurochko and Huang 1999). Second, monocytes are the predominant infiltrating cell type found in infected organs (Booss *et al.* 1989). Third, monocyte associated viremia is a prerequisite for MCMV-associated pathogenesis (Collins *et al.* 1994; Stoddart *et al.* 1994). However, *in vivo* and *in vitro* studies have shown that HCMV infection of monocytes is non-permissive and restricted to IE gene expression indicating an abortive infection (Kondo *et al.* 1994; Fish *et al.* 1996). Further, the HCMV replication cycle takes several days to weeks to complete while monocytes have a short life span of up to 3 days in circulating blood. These observations create a puzzling senerio where monocytes are found at sites of infection and are present during infection but can not support productive viral replication. Recently, HCMV infection was found to induce monocyte differentiation into macrophages which can support HCMV replication (Smith *et al.* 2004). Consequently, monocytes could disseminate HCMV throughout the body where once in the tissue would differentiate into macrophages to allow for complete viral replication and progeny production.

1.4.6 The Immune Response

HCMV infections are kept under control by the immune system; however, because the viral genome enters a state of latency, complete clearance of the virus is rarely achieved. The lack of immunity to suppress HCMV infection results in severe disease that can be seen in immunosuppressed individuals such as AIDS patients, transplant patients and congenitally infected infants. The most severe cases are often restricted to those individuals with impaired cell-mediated immunity, indicating the importance of this branch of the immune response (see below).

A patient with NK cell deficiency had unusually severe symptoms in response to a HCMV infection highlighting the critical role that NK cells play in suppressing the infection (Biron *et al.* 1989). Furthermore, mice depleted of NK cells exhibit enhance viral replication in spleen, liver and lung when infected with MCMV (Tay *et al.* 1998).

CD8+ and CD4+ CTLs are also involved in controlling the replication of HCMV and clearing the infection. The majority of CTLs are specific for pp65 matrix

phosphoprotein introduced to the host during viral entry (McLaughlin-Taylor *et al.* 1994; Beninga *et al.* 1995) although there are low levels of CTLS directed against gB, gH, IE72 and IE86 (Alp *et al.* 1991; He *et al.* 1995).

The humoral branch of the immune system appears to play a supportive role in keeping viral loads low rather than in clearance of HCMV. Mice deficient in immunoglobulin synthesis were able to clear to virus (Jonjic *et al.* 1994); however, mice immunized against MCMV gB protected against lethal challenge (Rapp *et al.* 1992). Intrauterine infection is less severe when vertical transmission occurs in pregnant women who have previously acquired HCMV (Tanaka *et al.* 1991; Fowler *et al.* 1992). Primary infection of immunosuppressed renal transplant patients results in more severe disease than those associates with reactivation of the virus (Plotkin *et al.* 1984). Consequently, it appears that hormonal immunity is important in limiting disease severity.

1.5 Vertical Transmission of Human Cytomegalovirus

1.5.1 Incidence

HCMV infection during pregnancy can be defined as either primary or recurrent. Primary maternal HCMV infection occurs when the virus is initially acquired during pregnancy; recurrent maternal HCMV infection, which includes both reactivation of the woman's own strain acquired previously and re-infection with a new strain of virus, is defined as the presence of maternal antibody to HCMV before conception. The rate of transmission to the fetus in primary maternal HCMV infection is 40% (Stagno *et al.* 1984); whereas, the rate of transmission for recurrent maternal infection averaged 1% (Stagno *et al.* 1982). It appears that acquisition of a new strain of HCMV contributes to the majority of vertical transmission for recurrent maternal infection (Boppana *et al.* 2001). Congential HCMV infection is unquestionably one of the most common congenital infections in the world occurring in 0.5% to 2.3% of all live births (Stagno *et al.* 1982; Fowler and Pass 1991).

After pregnancy and delivery, HCMV can be frequently found in almost all bodily fluids, especially in breast milk and cervicovaginal secretions, and thus a source for perinatal and postnatal transmission of HCMV from mother to infant. Up to 53% of infants who are fed breast milk containing infectious virus and 57% of infants whose

mothers shed HCMV in their cervicovaginal secretions at the time of delivery are perinatally infected (Reynolds *et al.* 1973; Dworsky *et al.* 1983). Although these transmission rates are significant, perinatal and postnatal HCMV infections in infants are almost always asymptomatic.

1.5.2 Clinical Outcome Congenital Infection

Fetal outcome, but not the rate of vertical transmission, is dependent on when the infection was acquired during pregnancy (Kumar and Prokay 1983; Yow *et al.* 1988). If fetal infection occurs earlier in pregnancy, the severity of the symptoms associated with congentital HCMV infection increases. Up to 10% of newborns with congenital HCMV infection will show immediate symptoms including death, jaundice, hepatosplenomegaly, and pneumonia (Demmler 1991; Nelson and Demmler 1997) (Fig 1.5). Effects on the central nervous system are also very common and include chorioretinitis, microcephaly, and sensorineural deafness. Nearly 5% of HCMV infected infants have unusual manifestations such as hemolytic anemia and chronic hepatitis (Demmler 1991). Long-term prospective studies indicate up to 80% of infants symptomatic at birth will exhibit lasting neurological abnormalities (Pass *et al.* 1980). Unfortunately, of the remaining 85% of congenitally infected infants born asymptomatic 15% will eventually suffer from hearing, vision, or developmental problems although the severity of the neurological impairment is much less then those seen in infants who are symptomatic at birth (Williamson *et al.* 1990).

1.5.3 Examination of Placentae from Congenitally Infected Fetuses

HCMV infection is a major cause of chronic placental villitis (inflammation) (Schwartz *et al.* 1992; Benirschke and Kaufmann 2000). Examinations of placentae from infants diagnosed with congenital HCMV infection often reveal villous destruction (Garcia *et al.* 1989; Greco *et al.* 1992) albeit the level of damage varied widely. The severity of villitis appears to be strongly associated with fetal outcome. Placental specimens from spontaneous macerated abortions showed severe diffuse villitis and a pronounced degree of dysmaturity (underdevelopment) of villous structures (Garcia *et al.* 1989). Placentas from liveborn infants whom were clinically normal at birth but

developed neurological abnormalities within the first few months of life showed less inflammation and a higher degree of differentiation of villous structures, although dysmaturity was still present. Microscopic examination of both groups revealed disruptions in the ST barrier through the formation of lesions. This ability of HCMV to infect and destroy trophoblast was demonstrated in placental explants (Amirhessami-Aghili *et al.* 1987).

1.5.4 Monocytes and Chronic Villitis

Accumulation and activation of monocytes/macrophages is a hallmark of chronic inflammation. TNFα plays a central role in the development of chronic inflammation and is secreted in large amounts by monocytes/macrophages (Vassalli 1992). There are twice as many monocytes per unit volume of blood in the intervillous space as in peripheral blood (Moore *et al.* 2003) suggesting a low affinity interaction between monocytes and the villous ST. The major infiltrating leukocytes in villitis associated with HCMV or with villitis of unknown etiology are mononuclear phagocytes (Altemani 1992; Greco *et al.* 1992) further increasing the number of monocytes found in the intervillous space. There is an accumulation of mononuclear phagocytes at sites of trophoblast loss (Garcia *et al.* 1989; Greco *et al.* 1992). In culture the inflammatory cytokines IL1, TNFα and IFNγ can stimulate monocyte adhesion to the ST surface by upregulating ICAM-1 (Xiao *et al.* 1997). IFNγ-activated ST cultures bind monocytes which induce trophoblast damage in a TNFα-dependent manner (Garcia-Lloret *et al.* 2000) suggesting that villous ST upregulation of ICAM-1 is an early step in villitis.

1.5.5 HCMV, chronic villitis and IUGR

In addition to the neurological damage caused by fetal infection, hematogenous infection of the placenta by HCMV is a major risk factor for fetal IUGR (Garcia 1982). Indeed, 25% of neonates display IUGR when the mother acquires a primary HCMV infection later during pregnancy (Istas *et al.* 1995). Although there are several possible origins of IUGR, all lead to deficient oxygen and nutrient delivery by the placenta to the fetus (Robinson and Owens 1996). Villitis characterizes placental infections by HCMV (Benirschke *et al.* 1974; Garcia *et al.* 1989; Schwartz *et al.* 1992; Benirschke and

Kaufmann 2000), is a risk factor for IUGR (Salafia *et al.* 1995) and is accompanied by focal damage to the villous trophoblast, the major function of which is nutrient delivery from maternal to fetal circulation (Benirschke and Kaufmann 2000). Thus, villous trophoblast damage by HCMV likely contributes to IUGR; however, the mechanism of damage is unknown.

1.5.6 Involvement of Placental Trophoblasts in Vertical Transmission

Spontaneous abortion occurs in approximately 15% of pregnant women with primary HCMV infection (Istas *et al.* 1995). In these cases placental, but not fetal, infection was evident suggesting that placental infection precedes virus transmission to the fetus (Hayes and Gibas 1971; Benirschke *et al.* 1974; Mostoufi-zadeh *et al.* 1984). Immunohistochemical analysis of sections from term placentas displaying chronic villitis revealed IE (Muhlemann *et al.* 1992; Sinzger *et al.* 1993) but not E (Muhlemann *et al.* 1992) or L (p150) (Sinzger *et al.* 1993) antigens, suggesting abortive infections (Sinzger *et al.* 1993). However, *in situ* hybridization revealed HCMV DNA in stromal cells and in the trophoblast of term placentae with chronic villitis (Sachdev *et al.* 1990; Kumazaki *et al.* 2002). Placentae from first or second trimester abortions contain nuclear inclusions frequently in stromal cells (Schwartz *et al.* 1992) and more rarely in trophoblasts (Garcia *et al.* 1989) with expression of early antigen pp65 in the trophoblast (van Lijnschoten *et al.* 1994; Halwachs-Baumann *et al.* 1998) suggesting some permissive trophoblast infection. *In vitro* cultured term villous trophoblasts can be productively infected in culture (Halwachs-Baumann *et al.* 1998; Hemmings *et al.* 1998)}; however, there does not appear to be any basal release of HCMV progeny particles from polarized cultured villous trophoblast (Hemmings and Guilbert 2002). Thus, how HCMV bypasses the ST barrier remains unclear.

There appear to be two potential routes of vertical transmission: 1) Tissue sections of villous explants challenged with HCMV for 4 days express IE proteins in the CT and extremely rarely in the ST (Fisher *et al.* 2000). Chorionic villi that were infected with HCMV *in utero* show a similar pattern of infection where the ST were often spared and the CT express a number of viral proteins. To account for these observations, it has be suggested that infection bypasses the ST via an extravillous route where HCMV can

spread through decidual cells from a uterine reservoir into the columns of CT situated below the villous placenta and into the villous stroma (Fisher *et al.* 2000; Pereira *et al.* 2003; McDonagh *et al.* 2004). Any infection of the ST would be rare and likely to occur later in the infection process (Fisher *et al.* 2000). 2) Alternatively, morphological studies showed that the focal inflammation associated with congenital HCMV infection is accompanied by the loss of the trophoblast resulting in the formation of lesions in the ST (Benirschke *et al.* 1974; Garcia *et al.* 1989; Schwartz *et al.* 1992; Sinzger *et al.* 1993). Placental explants indicated HCMV-induced histopathologic lesions bearing viral antigens were consistently localized in the trophoblastic cells covering placental villi (Amirhessami-Aghili *et al.* 1987). Thus, another potential route of vertical transmission could have HCMV directly bypassing the ST barrier through sites of damage. Regardless of the route of transmission, both must accommodate the phenomenon of placental villitis that accompanies vertical transmission of HCMV.

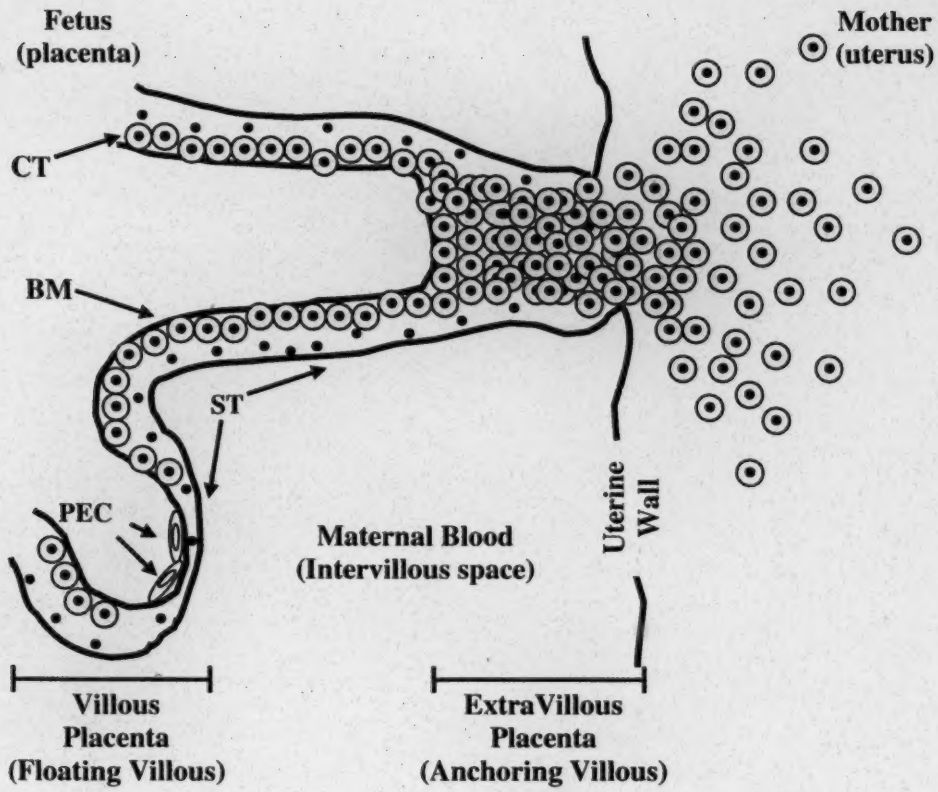

Figure 1.1: Diagram of the uterine placental interface at midgestation.

Extravillous CTs invade into the uterine wall to form the anchoring villous which connects fetal tissue to maternal tissue. The floating villous is bathed in maternal blood and is responsible for mediating the exchange of nutrients and oxygen between the maternal and fetal circulations.

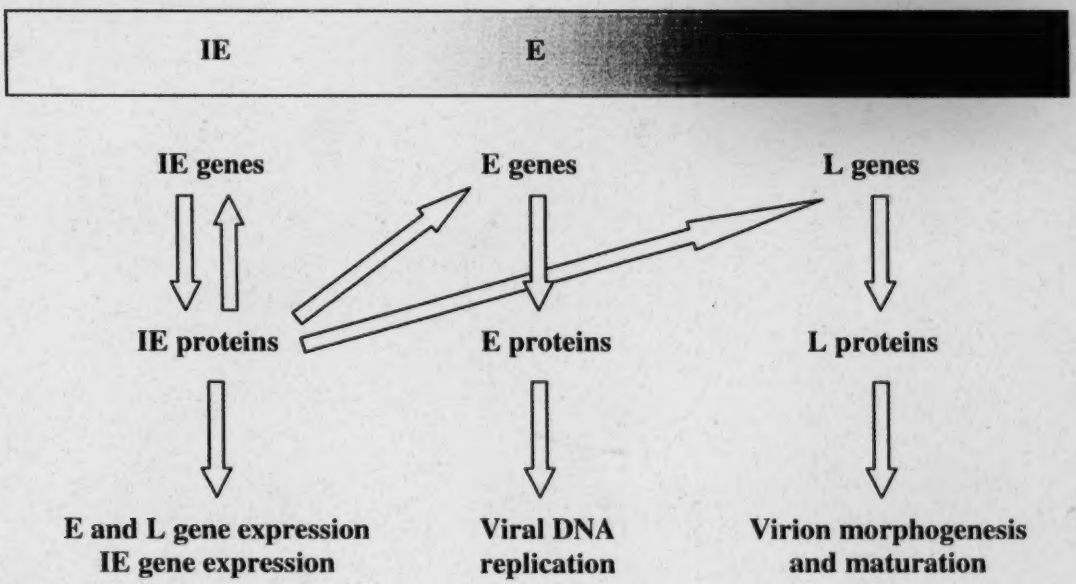

Figure 1.2: HCMV gene expression and viral gene products during productive infection.

HCMV gene expression occurs in sequential phases designated IE, E, and L. IE gene expression is required for the transcription of E genes, which encode proteins essential for viral DNA replication. In turn, replication of viral DNA is a prerequisite for L viral gene transcription of structural proteins.

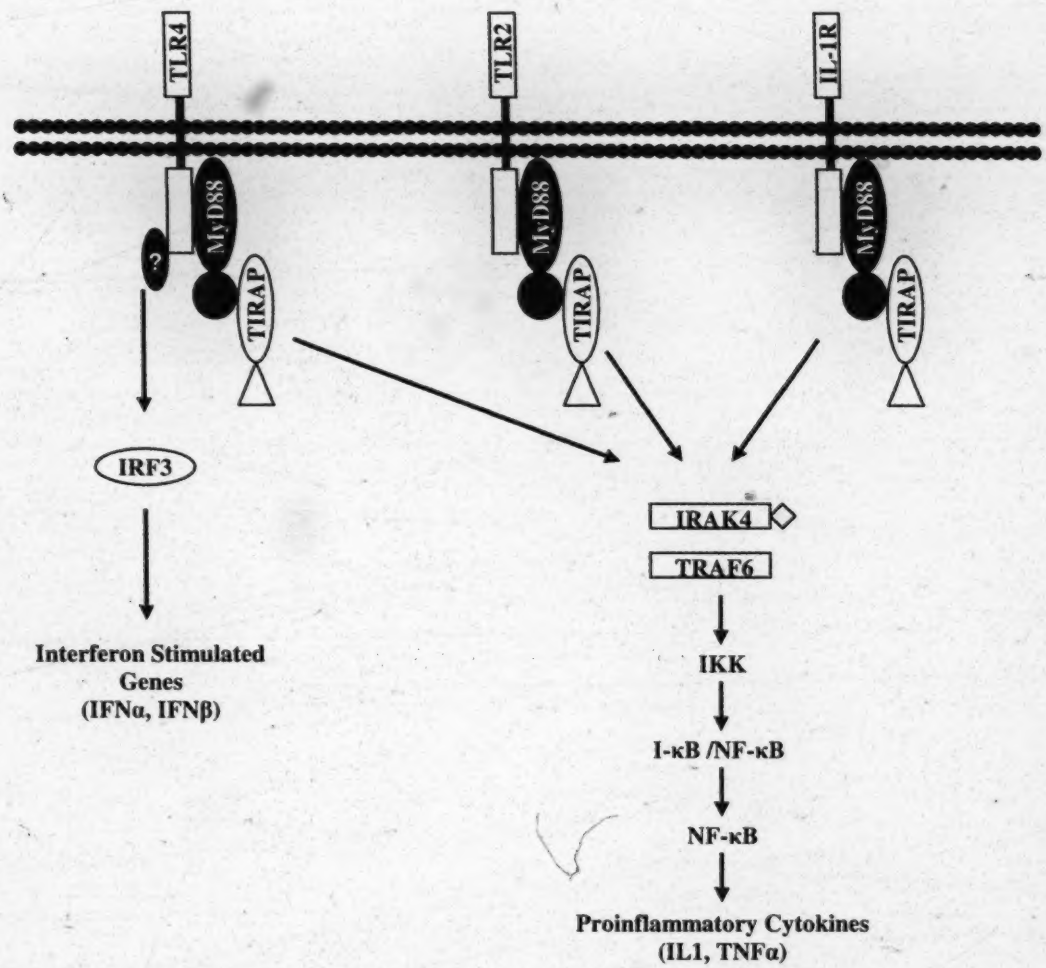

Figure 1.3: TLR and IL1R signaling pathway.

Upon activation with the appropriate ligand, both receptor types are able to recruit MyD88 which leads to the activation of NF-κB and the production of various inflammatory cytokines. Activation of certain TLRs are able to induce IFN stimulated genes via IRF3.

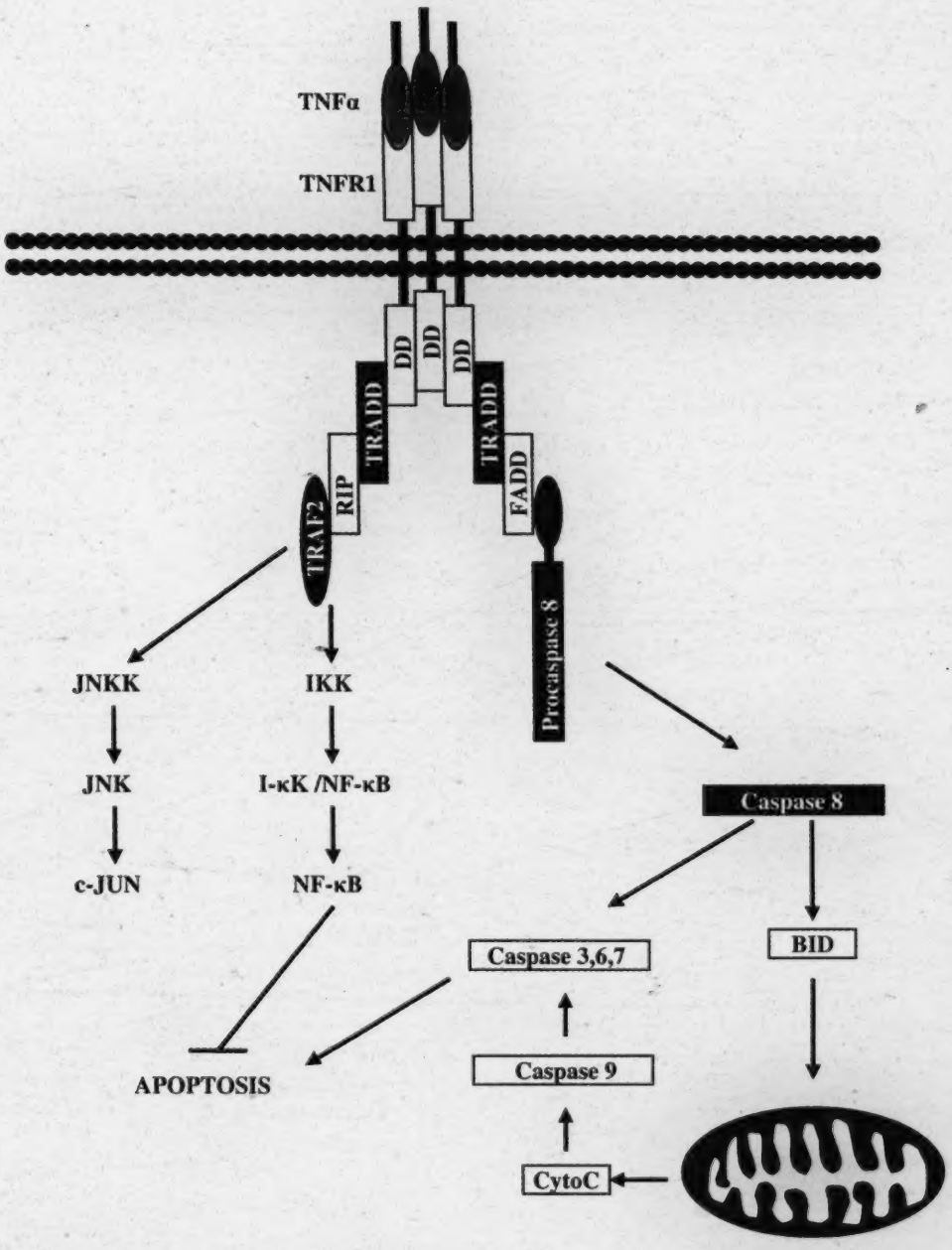

Figure 1.4: TNFR1 mediated signaling pathway.

Binding of TNFα to TNFR1 results in recruitment of various adaptor molecules to the DD. Recuritment of FADD cleaves and activates caspase 8 which, in turn, leads to the processing of effector caspases. Stimulation of TNFR1 also activates NF-κB and JNK signaling pathways via RIP and TRAF2, respectively.

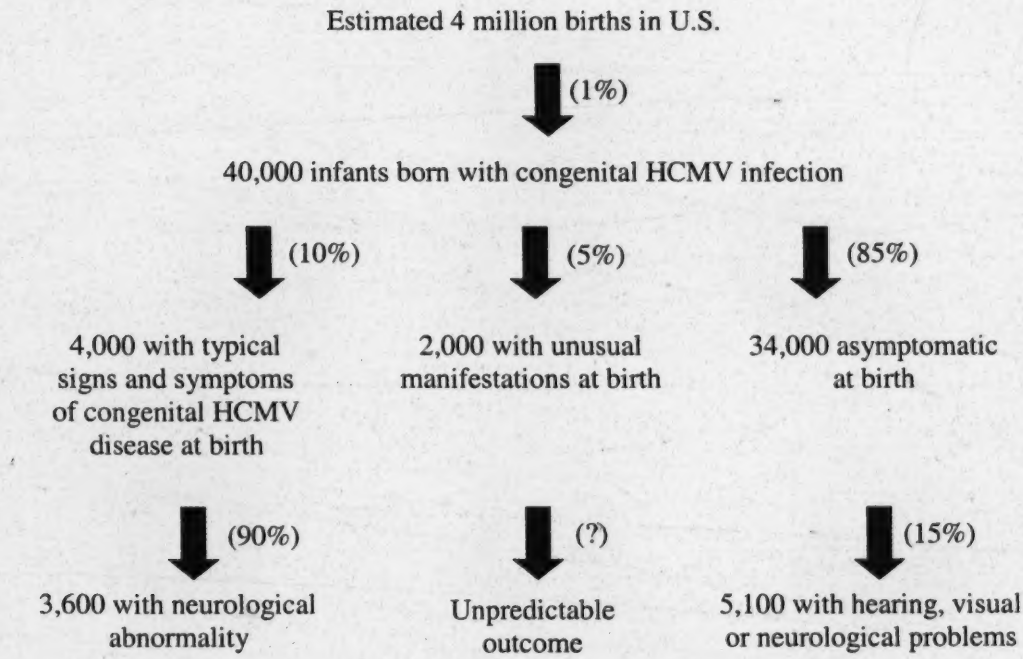

Figure 1.5: Clinical Outcomes of Congenital HCMV Infection

See Chapter 1.5.2 for further detail.

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CHAPTER 2: RATIONALE AND HYPOTHESIS

2.1 Hypothesis

HCMV infection of newborns occurs in approximately 2% of all live births making HCMV the most common congenital infection in the world (Stagno *et al.* 1982; Fowler and Pass 1991). Congenital HCMV is responsible for a number of developmental problems including mental retardation, cerebral palsy and sensorineural deafness (Demmler 1991). In addition to the neurological abnormalities, infection of the placenta can result in 25% of neonates exhibiting IUGR (Istas *et al.* 1995). Spontaneous abortion occurs in approximately 15% of pregnant women with primary HCMV infection (Istas *et al.* 1995). Placental, but not fetal, infection was evident suggesting that placental infection precedes vertical transmission (Hayes and Gibas 1971; Benirschke *et al.* 1974; Mostoufi-zadeh *et al.* 1984). The substantial medical impact and considerable expense to take care of congenitally infected and IUGR infants make understanding disease pathogenesis at the placental level of the utmost importance.

Passage of HCMV across the placenta can potentially occur in two routes. First is up through the cell columns in the anchoring villi (Fisher *et al.* 2000). The infection would originate in the uterine wall and spread to the extravillous CTs. HCMV could then migrate up the anchoring villi bypassing the ST and eventually infect the fetus. The second route is across the villous placenta. Infection and cell-to-cell spread across the ST is unlikely to occur since progeny viruses are released from polarized trophoblast only apically (i.e. towards the maternal circulation) (Hemmings and Guilbert 2002). However, HCMV infected placentae exhibit widespread villitis and the presence breaks in the ST barrier due to trophoblast loss (Benirschke *et al.* 1974; Garcia *et al.* 1989; Schwartz *et al.* 1992; Sinzger *et al.* 1993) suggesting an alternative possibility: HCMV transmission across the ST could occur through sites of damage. Moreover, damage to the ST layer could impair oxygen and nutrient deliver to the fetus resulting in IUGR. **My hypothesis is that HCMV infection of villous ST results in chronic villitis and villous trophoblast loss allowing for vertical transmission across sites of damage and decreased villous placenta function leading to IUGR.**

2.2 Rationale

I decided to study vertical transmission of HCMV from mother to fetus for the following reasons:

1. HCMV is the most common viral infection acquired during pregnancy occurring in up to 2% of all live births. Birth defects caused by congenital HCMV infections represent a major public health problem. Indeed, congenital HCMV infection is often responsible for sensorineural loss and is second only to Down syndrome as an identifiable cause of mental retardation. The long-term neurological developmental disabilities produced by infection of newborns uses a significant amount of resources in terms of therapy and patient care.

2. Placentae obtained from congenitally infected infants exhibit inflammation (villitis) resulting in trophoblast loss and the formation of lesions across the ST barrier. Breaks in the ST layer may serve as a conduit for vertical transmission; however, HCMV-induced trophoblast cell loss is not well understood.

3. Placental injury may compromise the development of the fetus even in the absence of vertical transmission due to placental dysfunction. Chronic villitis is a commonly associated with IUGR and HCMV is a common cause of villitis yet little is known about the mechanism by which HCMV induces inflammation.

4. The Towne vaccine, a live attenuated HCMV vaccine, has been evaluated in clinical trials. However, because of the lack of knowledge of vertical transmission, there is concern about the safety of such live virus vaccines if inadvertently administered to a pregnant woman.

5. This lab developed a technique to isolate highly purified human primary trophoblast (>99.99%). Villous CT cultures are predominately mononuclear and can be induced to form syncytium with the addition of EGF. These cultures express higher levels of differentiation markers such as placental alkaline phosphatase (PLAP) and hCG then CT cultures and thus resemble the mature ST. Trophoblast cells lines are generally not the best model for villous trophoblast because they proliferate and express histocompatibility antigen (HLA) class I molecules. Consequently, our primary trophoblast cultures make an excellent model for studying HCMV pathogenesis.

2.3 Virus Strains

The majority of experiments were done with HCMV laboratory strain AD169. AD169, isolated from the adenoids of a 7 year girl and passaged over fifty times in HF cells, was initially developed as a potential live attenuated vaccine (Prichard *et al.* 2001). Controlled human trials showed it to be avirulent in HCMV seronegative volunteers (Prichard *et al.* 2001).

Clinical isolate, Kp7, was obtained from a congenitally infected infant from Dr. J. Preiksaitis (Department of Medicine, University of Alberta). Kp7 has been passaged seven times in HEL cells.

Recombinant strain, RVdlmwt-green fluorescent protein (GFP) HCMV, was generiously donated by Dr. M. Stinski and described in (Isomura and Stinski 2003). Briefly, the UL127 gene was replaced with the GFP gene inserted downstream of the early UL127 promoter. Deletion and insertion of the GFP gene did not affect the growth kinetics when compared to parental Towne virus (Meier and Pruessner 2000).

Recombinant strain, UL32-enhanced GFP (EGFP) HCMV, was generously donated by Dr. C. Sinzger and described in (Sampaio *et al.* 2005). UL32-EGFP HCMV was generated by fusing the EGFP gene to the C terminus of the capsid-associated tegument protein pUL32 (pp150) open reading frame. Fluorescence could be monitored throughout viral entry and fluorescent progeny particles could be observed 44 hours post infection. Recombinant virus exhibited the same growth kinetics as parental strain TB40.

2.4 Experimental Design

My initials studies were to determine if HCMV infection of cultured trophoblast resulted in cell loss that could be assess by total nuclei count. These experiments showed significant trophoblast loss from infected cultures (Chapter 3). Consequently, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) staining was carried out to evaluate if apoptosis was involved. HCMV was found to induce apoptosis within the first 24 hours of infection suggesting early viral replication events play a pivotal role. Using plasmids expressing HCMV IE proteins, I found that IE protein expression could induce trophoblast apoptosis.

In addition, I found that challenge with transcriptionally silent UV-inactivated HCMV could also lead to ST death (Chapter 4). UV-inactivated HCMV stimulated the secretion of TNFα, as determined by ELISA, which was responsible the induction of apoptosis. Using neutralizing antibody to TLR2, I found that UV-inactivated virus particles stimulated the secretion of TNFα in a TLR2-dependent manner.

My third objective was to determine if HCMV infected ST expressed elevated levels of ICAM-1. These experiments, described in Chapter 5, led to the finding that HCMV stimulated surface ICAM-1 expression by a number of different mechanisms. Therefore, I next investigated monocyte adhesion to HCMV infected ST cultures in Chapter 6. ST infected cultures were co-cultured with primary monocytes and examined for monocyte binding by immunofluorescent staining for CD45 (expressed by monocytes but not ST). Infected cultures were found to have enhanced affinity for monocytes. Subsequently, the effects of increased monocyte adhesion on ST apoptosis was determined by TUNEL staining. Adherent monocytes were found to further elevate the levels of apoptosis when compared to infected ST alone.

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CHAPTER 3: HUMAN CYTOMEGALOVIRUS-CAUSED DAMAGE TO PLACENTAL TROPHOBLASTS MEDIATED BY IMMEDIATE-EARLY GENE INDUCES TUMOR NECROSIS FACTOR α

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3.1 Introduction

HCMV, a member of the *Herpesviridae* family, is a common infection found in 50-90% of adults (Fowler and Pass 1991). A primary infection is asymptomatic in immunologically healthy women but fetal infection by maternal HCMV is a serious problem. There is a 30-40% possibility of intrauterine transmission of HCMV to the fetus during a primary infection of the mother (Stagno *et al.* 1984) and a 0.2% - 0.5% possibility during a recurring or re-infection (Stagno *et al.* 1982). The overall frequency is 0.5 - 2% of all live births making HCMV the most common congenital infection (Stagno *et al.* 1982; Fowler and Pass 1991). A congenital HCMV infection can result in abortion or stillbirth with symptomatic survivors displaying sequalae such as thrombocytopenia, hepatosplenomegaly, vision loss, sensorineural deficits and mental retardation (Demmler 1991; Nelson and Demmler 1997).

HCMV can establish productive, persistent or latent infections in a variety of cells including those of epithelial origin [reviewed in (Mocarski 1996)]. HCMV gene expression occurs in sequential phases designated IE, E, and L. IE gene expression is required for the transcription of E genes, which encode proteins essential for viral DNA replication. In turn, replication of viral DNA is a prerequisite for L viral gene transcription of structural proteins. Transcription of IE genes has been mapped to five regions on the human HCMV genome. The most abundantly transcribed IE region is the ie1/ie2 locus. This region encodes two viral proteins, IE1-72 and IE2-86, and IE2-55, a splice variant of IE2-86. The IE1-72 protein interacts with the cellular transcription factors NF-κB, c-feline osteosarcoma (c-fos) and c-myelcytomatosis oncogene (c-myc) (Hagemeier *et al.* 1992; Yurochko *et al.* 1995; Johnson *et al.* 1999). The IE2-86 protein is a strong transcriptional activator that interacts with basal-transcriptional machinery and

blocks cell cycle progression (Murphy *et al.* 2000). Importantly, IE1-72 and IE2-86 regulate both viral gene and cellular gene expression. Through interactions with cellular factors such as p53 tumor-suppressor protein (p53) and NF-κB, both viral proteins inhibit apoptosis (Muganda *et al.* 1994; Speir *et al.* 1994; Tsai *et al.* 1996) and the induction of apoptosis by TNFα (Zhu *et al.* 1995).

In addition to the neurological damage caused by fetal infection, hematogenous infection of the placenta by HCMV is a major risk factor for fetal IUGR (Garcia 1982) that in turn is linked to cardiovascular disease later in life (Barker *et al.* 1989; Barker *et al.* 1990). Although there are several possible origins of IUGR, all lead to deficient oxygen and nutrient delivery by the placenta to the fetus (Robinson and Owens 1996). Villitis (inflammation of the villous placenta) characterizes placental infections by HCMV (Garcia *et al.* 1989), is a risk factor for IUGR (Salafia *et al.* 1995) and is accompanied by focal damage to the villous trophoblast, the major function of which is nutrient delivery from the maternal to the fetal circulations (Benirschke and Kaufmann 2000). Thus, villous trophoblast damage by HCMV likely contributes to IUGR; however, the mechanism of damage is unknown.

Immunohistochemical analysis of sections from term placentae displaying chronic villitis revealed IE (Muhlemann *et al.* 1992; Sinzger *et al.* 1993) but not E (Muhlemann *et al.* 1992) or L (p150) (Sinzger *et al.* 1993) antigens, suggesting abortive infections (Sinzger *et al.* 1993). However, *in situ* hybridization revealed HCMV DNA in stromal cells and in the trophoblast of term placentae with chronic villitis (Sachdev *et al.* 1990). Placentae from first or second trimester abortions contain nuclear inclusions frequently in stromal cells (Schwartz *et al.* 1992) and more rarely in trophoblasts (Garcia *et al.* 1989) with expression of early antigen pp65 in the trophoblast (van Lijnschoten *et al.* 1994; Halwachs-Baumann *et al.* 1998) suggesting a permissive trophoblast infection. Importantly, pure populations of term and first trimester villous trophoblasts can be productively infected in culture (Hemmings *et al.* 1998), observations that both confirm villous trophoblast infection and provide a model for studying HCMV-induced placental damage.

The villous trophoblast comprises two cell types: an extended syncytium spanning the intervillous surface (the ST) and an underlying layer of mononuclear CT (Benirschke

and Kaufmann 2000). Villous trophoblast apoptosis is a normal event in placental development (Smith *et al.* 1997) that is increased in placentas associated with IUGR (Smith *et al.* 1997). Primary villous trophoblast apoptosis in culture occurs spontaneously and is stimulated by the inflammatory cytokines TNFα and IFNγ (Yui *et al.* 1994; Garcia-Lloret *et al.* 1996) and by serum-withdrawal (Desai *et al.* 1999). Primary villous CT can be isolated (Guilbert *et al.* 2002) and differentiated in culture into syncytialized clusters by treatment with EGF (Garcia-Lloret *et al.* 1996; Morrish *et al.* 1997). Thus, cultures of mature and immature primary villous trophoblasts provide a good model for investigating the consequences of villous trophoblast infection by HCMV.

Using this model, we asked whether HCMV infection damages trophoblast cultures, and if so, how. We found that within 24 hrs of infection over half of both mature and immature trophoblasts were lost by apoptosis stimulated by TNFα release but that only uninfected cells in the culture underwent apoptosis. We also show that expression of HCMV immediate early genes IE1-72 and IE2-86 are sufficient for these effects.

3.2 Methods and Materials

3.2.1 Cells

Human embryonic lung fibroblasts (HEL) were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and propagated in Eagles' minimum (MEM) supplemented with 10% fetal bovine serum (FBS, GIBCO, Grand Island, NY) and 50 μg of gentamicin per ml. L929-8 cells were maintained in culture with Iscove's Modified Dulbecco's medium (IMDM; GIBCO) containing 15% FBS as previously described (Branch *et al.* 1991).

Human term villous CT were isolated from placentae obtained after normal term delivery or elective cesarean section from uncomplicated pregnancies and cryopreserved as previously described (Yui *et al.* 1994; Kilani *et al.* 1997). After thawing, the cells were washed in IMDM supplemented with 10% FBS, seeded in 96 well dishes (NUNC, Roskilde, Denmark) at 10^5 per microwell per 100 μl 10% FBS/IMDM and incubated for 4 hrs at 37°C in a fully humidified atmosphere of 5% CO_2 in air. Nonadherent cells and

debris were washed away with prewarmed IMDM and the cultures continued in 10% FBS/IMDM. All preparations contained fewer than 10 vimentin-positive cells after the 4-hr wash. All experiments were carried out either with cells cultured for 24 hrs without EGF (operationally termed CT cultures) or with cells that had been syncytialized by treatment with 10 ng/ml EGF (Prepro-Tech, Rocky Hill, NJ) for 5 days as previously described (Garcia-Lloret *et al.* 1996) (operationally termed ST cultures).

The adherence and spontaneous apoptosis of cryopreserved primary cytotrophoblasts from a single placenta are consistent in independent experiments carried out at different times. However, these properties can be very different in cells from different placentas even if examined at the same time. Each experimental data set (figure) consists of three independent experiments with cells from two different placentae (two experiments with one placental preparation and one from the other). Six different placental preparations were used in the experiments. The two placental preparations for a given figure were chosen for a single consistent property (the fraction of cells that adhered after 4 hrs of culture) before the experiments were carried out. The two placental preparations, thus chosen, showed a consistent basal frequency of apoptosis, either high or low, and HCMV infection consistently increased this basal frequency irregardless of whether it was high or low. For example, even though spontaneous apoptosis frequencies for CT and ST cultures varied considerably (1-17%) between Figs 3, 5, 6 and 7, the ratios of HCMV-induced to basal apoptosis frequencies across these experimental groupings were consistent (2.4 ± 0.7, N=8). Thus, the trends are consistent for cells from all placentae, even though absolute numbers may be different.

3.2.2 Virus preparation, culture challenge and assessment of infection

HCMV laboratory strain AD169 was passaged in confluent HEL cells in 2%FBS-MEM as previously described (Hemmings *et al.* 1998), the lysate passed through 0.45-μm-pore-size filters (MILLEX-HV; Millipore Products Division, Bedford, MA) and stored in liquid nitrogen until use. Viral titers were determined by inoculating confluent HEL cultures in 96-well plates with dilutions of each virus preparation in serum-free MEM. The plates were then centrifuged for 45 min at 2,500 rpm in a GCL-2 Sorvall centrifuge, the wells washed five times with warm MEM and the plates incubated for a

further 18 to 20 hrs in fresh 2% FBS-MEM. The cultures were fixed in ice-cold methanol and immunohistochemically stained for HCMV IE antigen as described below. Each IE-positive nucleus was equated to an infection focus (IF) of infectious virus, and the titer of virus was determined within a linear dose-response concentration range as IF/mL.

Where indicated HCMV preparations were inactivated by exposure to UV-light (30 W, Germicidal, distance of 20 cm) on ice for 20 min. Virus-free supernatant was obtained by filtering HCMV batches through a 0.1 μm pore size syringe top filter (Millipore). UV inactivation and complete filtration was assured by the absence of IE positive nuclei in trophoblast cultures.

Modifications in HCMV challenge methods have increased trophoblast infection frequencies considerably compared to a previous publication (Hemmings *et al.* 1998). The modified methods are summarized below: ST cultures were virus challenged five days after plating and CT trophoblasts one day after plating. Both culture types were washed once with warm IMDM and challenged in 2% FBS/IMDM for six or 24 hrs. The virus challenge was at a multiplicity of infection (MOI) of ten, calculated by first enumerating, in parallel cultures, the number of nuclei in CT and ST cultures by 4,6-diamidino-2-phenylindole (DAPI; Molecular Probes) staining (see below) and then adding a ten-fold higher IF of virus (or an equal volume of UV-inactivated or virus-free supernatant from the same preparation). After the six or 24 hr culture, the cells were washed twice with phosphate-buffered saline (PBS), fixed in ice-cold acetone:methanol (1:1) for 10 min at –20°C, and washed three times with PBS in preparation for immunofluorescence and/or TUNEL [terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin DNA-nick end labeling (Gavrieli *et al.* 1992)] (see below). Cultures extending longer than 24 hrs were washed at that time five times with warm IMDM, fresh 2% FBS-IMDM with (for ST cells) or without EGF (for CT cells) added and the media changed every two days for the duration of the culture. In some experiments (i.e. Fig 6), EGF was added to both culture types during the virus challenge period.

3.2.3 Transfection of HCMV IE expression plasmids

All plasmids were propagated in Escherichia coli DH5α, isolated by standard procedures and the plasmid DNA purified with a Qiagen Plasmid Maxiprep kit (Mississuaga, Ontario, Canada). Plasmids pcDNA3-IE1-72, pcDNA3-IE2-55, and pcDNA-IE2-86 respectively express the HCMV IE proteins, IE1-72, IE2-55, and IE2-86 (Yurochko *et al.* 1995). The vector without inserts, pcDNA3 (Invitrogen; San Diego, CA), served as a negative control. Trophoblasts (both CT and ST cultures) were prepared in microwells as described above and transfected with Lipofectamide 2000 (Gibco)/plasmid DNA complexes as follows: 700 μg of DNA in 25 μl Opti-MEM (Gibco) was mixed with 0.5 μl of 1 mg/ml Lipofectamine 2000 diluted with 25 μl of Opti-MEM then added to a microwell containing 100 μl of 2% FBS/IMDM. The transfection efficiencies for each plasmid type were determined by IE immunofluorescence as described above.

3.2.4 Immunofluorescence staining

Three-color fluorescence analysis was carried out to determine total nuclei number, the fraction and location of nuclei expressing HCMV IE proteins, and the fraction and location of nuclei containing nicked double-stranded DNA (a marker of apoptosis). After acetone:methanol fixation, and PBS washing, the fraction of nuclei with nicked DNA was determined by TUNEL as previously described (Yui *et al.* 1994) with modifications to allow for simultaneous immunofluorescence analysis. After the reaction was terminated by adding double-strength 300 mM sodium chloride plus 30 mM sodium citrate (2x SSC), the cells were washed three times with double distilled water, non-specific binding sites blocked with 3% skim milk/0.5% Tween 20/PBS for 30 min and primary antibody to HCMV IE (detecting p72; Specialty Diagnostics, Dupont) or its immunoglobulin (Ig)G1 isotype control (Dako Corporation, Carpinteria, CA) added and incubated at room temperature for 1 hr. The primary antibody was then removed, the cells washed five times with PBS and 50 μl per well of streptavidin Alexa Fluor 488 conjugate (Molecular Probes, Eugene, OR) and Alexa Fluor 546 goat anti-mouse IgG conjugate (Molecular Probes) each diluted in 3% skim milk/0.5% Tween 20/PBS to 1 μg/ml added and incubated for 1 hr at room temperature. The cells were then washed

five times with PBS. To visualize all nuclei 100 µl of 1.4 µg/ml DAPI (Molecular Probes) was added to each well and allowed to sit for 10 min at room temperature. Cells were then washed with PBS five times and visualized with a fluorescence microscope (see below). The total number of nuclei (DAPI, blue), IE positive (Alexa Fluor 546, red) and TUNEL positive (Alexa Fluor 488, green) were determined per well by digital analysis as described below.

3.2.5 Digital photography and analysis

Flourescence was visualized with an inverted phase contrast microscope (Model DS-IRB, Leica; Heerbrugg, Switzerland) equipped for epifluorescence with a 50 W high pressure mercury lamp driven by a Ludl power source (Ludl Electronic Products; Hawthorne, NY). Identical digital images of each well were taken with a DAPI filter (blue), a rhodamine filter (red) and a FITC filter (green) using a SPOT digital camera (Diagnostic Instruments; St Sterling Height, MI). The red and green images were superimposed using an imaging program, Image-Pro Plus (Media Cybernetics; Del Mar, CA). Three images, each containing ~ 900 nuclei, were taken in each of triplicate wells.

3.2.6 TNFα neutralization

To neutralize biologically active TNFα released in trophoblast cultures, 20 µg/ml of polyclonal anti-human TNFα antibody (anti-TNFα; ICN, Aurora, OH) was added to the culture at the time of virus challenge. Following incubations as noted in individual figure legends, cells were washed with PBS three times, fixed with acetone:methanol (1:1) and TUNEL analysis and immunofluorescence carried out as described above.

3.2.7 Statistical analysis

Experiments for each figure were performed at least 3 times on trophoblasts isolated from two different placentae. Differences between experimental groups for the two cell types (CT or ST cells) were evaluated by one-way ANOVA (analysis of variance) with pairwise multiple comparison procedures (Tukey Test) using the SigmaStat program (Jandel Scientific, San Rafael, CA). Results were considered to be significant at $P<0.05$.

3.3 Results

3.3.1 HCMV infection of trophoblast induces cell loss within 24 hrs of virus challenge

Both immature (CT) and mature (ST) trophoblasts were challenged with HCMV laboratory strain AD169 and the infection allowed to progress over an 11 day period (Fig 3.1 shows a typical infection time course). Cells were stained with DAPI to determine total nuclei per well and the frequency of infection was determined by immunostaining for HCMV IE antigens. Six hrs after virus addition there were few detectable IE positive nuclei in either culture type. However, 24 hrs after addition approximately 25% of nuclei in CT cultures were IE positive and 3% of nuclei in ST cultures. By day 11 of culture >70% of nuclei remaining in CT cultures were IE positive compared to approximately 10% of nuclei in the ST cultures.

Because of the differing proportions of multinucleated cells in CT and ST cultures (Morrish *et al.* 1997), cell loss was characterized in both culture types by counting nuclei and comparing infected to control cultures (Fig 3.2). Three negative control groups were examined: mock infected control (Control), virus preparations filtered free of virus particles (Filtered), and UV-inactivated virus preparations (UV-inactivated). The positive control was treated with the inflammatory cytokines TNFα and IFNγ, known to stimulate both cell loss and apoptosis (Yui *et al.* 1994; Garcia-Lloret *et al.* 1996). Six hours after challenge, for a given culture type, all groups contained the same number ($P>0.05$) of nuclei (average ± SD for CT-like cultures: $4.1 \times 10^4 \pm 1.2 \times 10^3$/well, for ST-like cultures $6.2 \times 10^4 \pm 4.2 \times 10^3$/well). However, 24 hrs after challenge both HCMV infected CT and ST cultures lost approximately half of their nuclei compared to untreated control cultures ($P<0.05$) (Fig 3.2). Treatment of both culture types with UV-inactivated and filtered HCMV preparations did not significantly affect nuclei numbers at 24 hrs compared to control cultures ($P>0.05$). TNFα/IFNγ-treated CT cultures lost almost sixty percent of nuclei but ST cultures lost fewer than 20%.

Apoptosis of trophoblasts remaining in culture after six and 24 hrs was measured as the fraction of nuclei having double-stranded DNA nicks as visualized by TUNEL analysis. Six hours after virus exposure, for a given culture type, all groups contained the same fraction of TUNEL-positive nuclei ($P>0.05$) (average ± SD for CT cultures: 3.95 ±

0.66 %, for ST cultures 1.5 ± 0.46 %). However, 24 hrs after virus exposure both HCMV infected CT and ST cultures showed a significantly increased apoptosis frequency compared to control cultures (from 3.3 to 8.3% for CT cultures and 0.94 to 3.5% for ST cells, $P<0.05$) (Fig 3.3). Treatment of both culture types with UV-inactivated and filtered HCMV preparations did not significantly increase the TUNEL frequency at 24 hrs compared to control cultures ($P>0.05$). Both TNFα/IFNγ-treated cultures had a significantly increased frequency of TUNEL positive nuclei compared to control cultures (from 3.3% to 14.6% for CT cultures and from 0.94 to 2.1% for ST cultures, $P<0.05$, Fig 3.3).

Taken together, these observations strongly suggest that trophoblasts are lost by accelerated apoptosis induced by HCMV infection. However, cell loss at 24 hrs in HCMV-infected cultures (~50%) is consistently much higher than the frequency of apoptosis (8.3 and 3.5% for CT and ST cells, respectively).

Because of the large difference between cell loss and apoptosis frequencies, we asked whether cells that were simultaneously infected and undergoing apoptosis might be preferentially lost in culture. Non-adherent cells were collected from HCMV-infected ST and CT cultures 24 hrs after HCMV challenge and evaluated for apoptosis by TUNEL analysis and for HCMV infection by IE immunofluorescence. Non-adherent cells from CT cultures were 98.3 ± 0.58% TUNEL positive and 6.1 ± 0.81 % IE positive while non-adherent cells from ST cultures were 96.1 ± 1 % TUNEL positive and 17.2 ± 7.0 % IE positive. Thus, almost all cells lost from the cultures were undergoing apoptosis and <20% were HCMV infected.

3.3.2 HCMV kills only uninfected cells in culture

The above results suggested that most of the cell loss in HCMV challenged cultures was in the uninfected population. This suggestion is supported by the data in Figs 3.1 and 3.2 showing that 24 hrs after virus challenge CT and ST cultures lose the same fraction of nuclei even though the infection frequency in CT cultures is ten-fold higher than in ST cultures. In order to spatially characterize the relationship between infected and dying cells, we carried out two-color immunofluorescence analysis of HCMV-IE expression (red in Fig 3.4A) and apoptotic nuclei (by TUNEL analysis, green

in Fig 3.4A) on cells remaining adherent in the cultures. Concurrent nuclear DNA nicking and IE expression would result in nuclei with a combined yellow color (see Fig 3.4B, CT panel for a very rare example). One day after exposure to virus, there were green and red, but almost no yellow, nuclei in both CT and ST cultures. Thus, HCMV-IE and TUNEL stained nuclei were in mutually exclusive populations in the adherent population. Thus, for at least five days after HCMV infection, only uninfected trophoblasts in the adherent cultures are undergoing apoptosis.

3.3.3 HCMV-induced trophoblast cell loss is mediated by TNF α

The above observations that infection increases trophoblast apoptosis but only in uninfected cells suggests that HCMV-induced apoptosis is mediated by a soluble factor released by infected cells. The most likely (and only known) death-inducing factor for primary villous trophoblasts is the cytokine TNFα (Yui *et al.* 1994; Garcia-Lloret *et al.* 1996). We therefore first asked whether HCMV-induced apoptosis could be blocked by neutralizing antibody to TNFα. This was tested by carrying out the cell loss and apoptosis experiments depicted in Figs 3.2 and 3.3 in the presence of excess neutralizing antibody to TNFα. We first determined that antibody treatment does not inhibit infection: 24 hrs after HCMV challenge 15 ± 0.35% of nuclei were IE positive in antibody-treated ST cultures compared to 16 ± 0.71% in controls and 32.2 ± 2.9% in antibody-treated CT cultures compared to 31.2 ± 3.8% in controls. We then asked whether the antibody inhibited infection-induced culture damage: both HCMV-induced cell loss (Fig 3.5A) and apoptosis (Fig 3.5B) for both CT and ST cultures were completely inhibited by TNFα antibody. These observations argue that HCMV-induced trophoblast loss and apoptosis is mediated by infection-induced release of TNFα.

Concomitant treatment with the growth factor EGF also completely inhibits TNFα-induced apoptosis of placental trophoblasts (Garcia-Lloret *et al.* 1996; Garcia-Lloret *et al.* 2000). As predicted of a TNFα-driven trophoblast apoptosis process, EGF completely inhibits HCMV-induced trophoblast apoptosis (Fig 3.6).

3.3.4 HCMV-induced trophoblast cell loss and apoptosis is mediated by viral IE1 and IE2 genes

The kinetics of HCMV-induced trophoblast loss and apoptosis suggest a very early event in viral replication to be responsible. The earliest event, virus coat protein interactions with trophoblast plasma membranes appears unlikely since UV-inactivated virus preparations fail to induce significant cell loss and apoptosis (although a slight increase was in apoptosis was seen in ST cultures infected with UV-inactivated HCMV) (Figs 3.2 and 3.3). We therefore asked whether transcription of viral IE genes might alone induce trophoblast damage.

Parallel cultures of CT and ST cells were individually transfected with mammalian expression plasmids carrying IE1-72, IE2-55 and IE2-86 genes driven by the HCMV IE promoter (Yurochko *et al.* 1995) or the empty vector plasmid or infected with HCMV at an MOI of 10, then cultured for 24 hrs. At this time, the frequencies of IE-positive nuclei in IE2-55, IE1-72, and IE2-86 transfected CT cultures were 24.6 ± 5.9%, 25.2 ± 4.5%, and 22.3 ± 1.8%, respectively, and for ST cultures 20.1 ± 0.6%, 22.5 ± 2.2%, and 20.8 ± 7.4%, respectively. These levels are comparable to CT expression levels at day 1 after infection with HCMV (see Fig 3.1). Cell loss and apoptosis were monitored as described above. The results show that transfection with empty plasmid does not decrease cell number or increase the apoptosis frequency for either culture type but that transfection with IE1-72 and IE2-86 genes resulted in a significant loss of cells (Fig 3.7A) and increase in apoptosis frequencies (Fig 3.7B) relative to the empty plasmid-transfected control. Even though all three IE genes were expressed at similar frequencies (see above), their effects on cell loss and apoptosis vary in the order IE1-72>IE2-86>IE2-55, with the latter showing elevated, but not statistically significant, effects (n=3 independent experiments).

The relationship of IE1-72 and IE2-86 expression and apoptosis was also examined by two-color immunofluorescence [for IE proteins and apoptosis (TUNEL)] 24 hrs after transfection (Fig 3.4B, note that Fig 3.4A and 3.4B were from different experiments with differing numbers of cell in the culture). Even with IE protein expression (red) and TUNEL expression (green) near 20%, overlapping expression (yellow) was never observed, suggesting paracrine death. As with HCMV-infected

trophoblasts, both CT and ST cell loss and apoptosis induced by IE genes were inhibited by neutralizing TNFα antibody (Fig 3.7 A and B). EGF also completely inhibited IE1-72 further supporting the conclusion that HCMV-IE gene induced apoptosis of neighboring cells is mediated by TNFα (Fig 3.6).

3.4 Discussion

Placental infections by HCMV are accompanied by villous inflammations (villitis), *in utero* transmission of the infection from mother to fetus and low birth weight babies [reviewed in (Fox 1993; Bernstein and Divon 1997)]. Villitis associated with placental infections are characterized by regional loss of the trophoblast lining of the villous placenta. Although villous trophoblast infection is seen *in vivo* (Garcia *et al.* 1989; Saehdev *et al.* 1990; van Lijnschoten *et al.* 1994) and primary villous trophoblasts can be infected in culture (Halwachs-Baumann *et al.* 1998; Hemmings *et al.* 1998) the crucial relationship between trophoblast infection and infection-related loss of the villous trophoblast barrier has never been investigated. We here show an almost 50% loss of cells from cultures of primary villous trophoblasts in the first 24 hrs after HCMV challenge. This loss occurs before progeny virus release [which occurs only after a week of culture (Hemmings *et al.* 1998)], suggesting that cell loss by progeny virus-induced cytolysis is not likely. On the other hand, our findings that cell loss is accompanied by parallel increases in apoptosis, that virtually all lost cells are undergoing apoptosis and that the anti-apoptotic agents TNFα antibody and EGF also inhibit infection-induced cell loss strongly argue that infection-induced apoptosis causes the loss. Further analysis of the coincidence of HCMV-IE expression and double-stranded DNA nicking in nuclei of cultured cells revealed that only uninfected cells undergo apoptosis while attached to the tissue culture dish. Thus, HCMV infection of villous trophoblast populations has two separate effects: it induces paracrine apoptosis of uninfected cells and it prevents autocrine apoptosis of infected cells. These results argue that a local HCMV infection of the placental trophoblast does not directly lead to its death but rather to a massive and rapid loss of neighboring trophoblast. We suggest that this secondary loss of trophoblast after infection strongly contributes to HCMV-related placental villitis.

Our data showing 96-98% of non-adherent cells to be TUNEL positive after HCMV infection with 6-17% being HCMV-IE antigen positive contrasts with the strict segregation of TUNEL-positive and infected cells in the adherent population. We suggest that the massive loss of cells from cultures in the first 24 hrs after HCMV challenge may slough areas of the culture containing both apoptotic and infected nonapoptotic cells. Because trophoblasts, like other epithelial cells, may undergo anoikis after disruption of adhesion (Frisch and Francis 1994), non-apoptotic infected cells in the sloughed populations may undergo apoptosis secondary to loss of adhesion.

Our data show that HCMV-induced apoptosis and cell loss occur within 24 hrs of infection and are thus early events in the virus life cycle. The earliest event in herpes virus-host cell interaction is induction of an interferon-like response induced by interaction of virus coat proteins with the plasma membrane and results in upregulation of, among other genes, NF-κB, a nuclear factor known to regulate TNFα transcription (Yurochko *et al.* 1995; May and Ghosh 1998; Boyle *et al.* 1999; Mossman *et al.* 2001). The finding in this paper that neutralizing antibody to TNFα completely inhibits infection-induced apoptosis of uninfected trophoblasts shows TNFα is required for HCMV-induced paracrine killing. However, UV-inactivated virus did not alone stimulate appreciable apoptosis or cell loss (marginal increases in ST cultures) suggesting that virus binding and internalization are not sufficient for the magnitude of death observed and that viral gene transcription and translation are required.

HCMV IE gene expression is the most rapid viral transcriptional event, with gene products appearing in primary trophoblasts within 24 hrs (see Fig 3.1). There are several HCMV IE genes, the most common of which map to the ie2/ie2 region of the viral genome and are translated as IE1-72, IE2-86 and IE2-55 proteins. These gene products promote expression of subunits of the host cell nuclear transcription factor NF-κB, which in turn mediates both TNFα transcription and resistance to TNFα-induced apoptosis (Yurochko *et al.* 1995; Van Antwerp *et al.* 1998). Our present results show that expression of IE1-72 and IE2-86 alone mimic the ability of whole virus to stimulate TNFα-mediated paracrine killing in the first 24 hrs after infection. This report confirms earlier observations in other cell types of a dual role for HCMV IE genes in upregulation of TNFα transcription and protection against TNFα-stimulated apoptosis in infected cells

(Zhu *et al.* 1995). However, this is the first report of a dual role for HCMV IE function in primary placental trophoblasts and, more generally, to the consequences of damage to uninfected neighboring cells in any tissue. This is also the first study of the expression and function of individual HCMV IE genes in primary trophoblasts and the first to document meaningful (>1%) levels of transfection of any plasmid into these cells.

TNFα is a centrally important cytokine in placental development (Hunt *et al.* 1996). It is produced in the villous placenta (Chen *et al.* 1991) and both p55 and p75 receptors are found on the trophoblast in vivo (Yelavarthi and Hunt 1993) and on cultured villous trophoblasts (Yui *et al.* 1996). The role of TNFα in placental development is not necessarily limited to apoptosis and may depend on a microenvironment that regulates apoptosis and allows other functions of activated p55 receptors (Natoli *et al.* 1998). Indeed, the cytotoxic effects of TNFα on primary trophoblasts can be inhibited by concomitant presence of EGF (Garcia-Lloret *et al.* 1996). Our observation that EGF inhibits HCMV-induced trophoblast loss and apoptosis further supports the conclusion that HCMV-induced trophoblast damage is mediated by TNFα and suggests that damaging effects of HCMV on the villous trophoblast may also be subject to microenvironmental regulation.

The ability of HCMV infection to induce paracrine killing of trophoblasts is a consequence of the rather slow virus replication cycle in these cells. HCMV IE gene products appear in primary trophoblasts within 24 hrs of challenge (see Fig 3.1) but infectious progeny virus is not released until several days after infection (Hemmings *et al.* 1998). Thus, TNFα secretion induced by IE gene expression precedes by days neighboring cell infection that could inhibit TNFα-induced apoptosis. Thus, a localized infection of the ST (the first step in a hematogenous infection of the placenta) could result in enhanced apoptosis of near-by unprotected (uninfected) ST and underlying CT. This would be predicted to both increase ST aging into syncytial knots and compromise the renewal capacity of the local trophoblast through CT apoptosis. These outcomes are in accord with placental pathologies associated with IUGR: a rat model is characterized by excessive TNFα expression (Miller *et al.* 1996) and human IUGR placentas are characterized by excessive villous trophoblast apoptosis (Smith *et al.* 1997) and enhanced syncytial knot formation (van der Veen and Fox 1983).

The present studies also have implications for mechanisms of hematogenous transmission of HCMV from mother to fetus across the placenta. An intuitive model of HCMV passage would have an infected villous trophoblast releasing progeny virus basally into fetal tissue. However, observations that productively infected villous trophoblasts release less than 5% of progeny virus in conventional cultures (Hemmings *et al.* 1998) do not support such an infection and release model. Further, the little progeny virus released from polarized ST membrane cultures is >99% apical (toward maternal, not fetal, circulation) (Hemmings and Guilbert 2002). Thus, an infection and basal release mechanism of placental passage of HCMV is not likely. However, the above-mentioned hypothesis that ST infection ultimately leads to regional loss of the trophoblast barrier presents a condition that would allow access of infected maternal leukocytes into the villous stroma. That *in utero* transmission of HCMV might be secondary to virus-induced trophoblast damage is in accord with correlations between vertical transmission of HCMV and placental villitis (Benirschke *et al.* 1974) and between placental infections by HCMV and IUGR (Bernstein and Divon 1997).

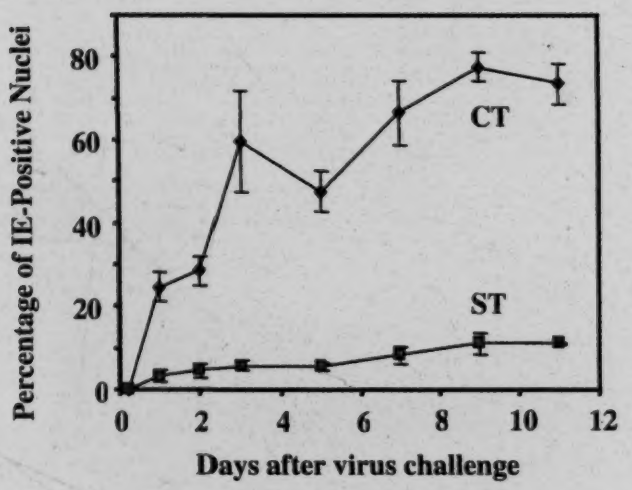

Figure 3.1: HCMV-IE protein expression in virus challenged trophoblasts as a function of time after challenge.

CT and ST cultures were challenged at an MOI of 10 with HCMV strain AD169 and cultured as described in the Methods and Materials. At the times indicated, IE-positive nuclei were visualized with an Alexa Fluor 547 conjugate and total nuclei with DAPI, as described in the Methods. Depicted is the mean ± SD of two independent experiments with two different placentae, each containing three replicate cultures.

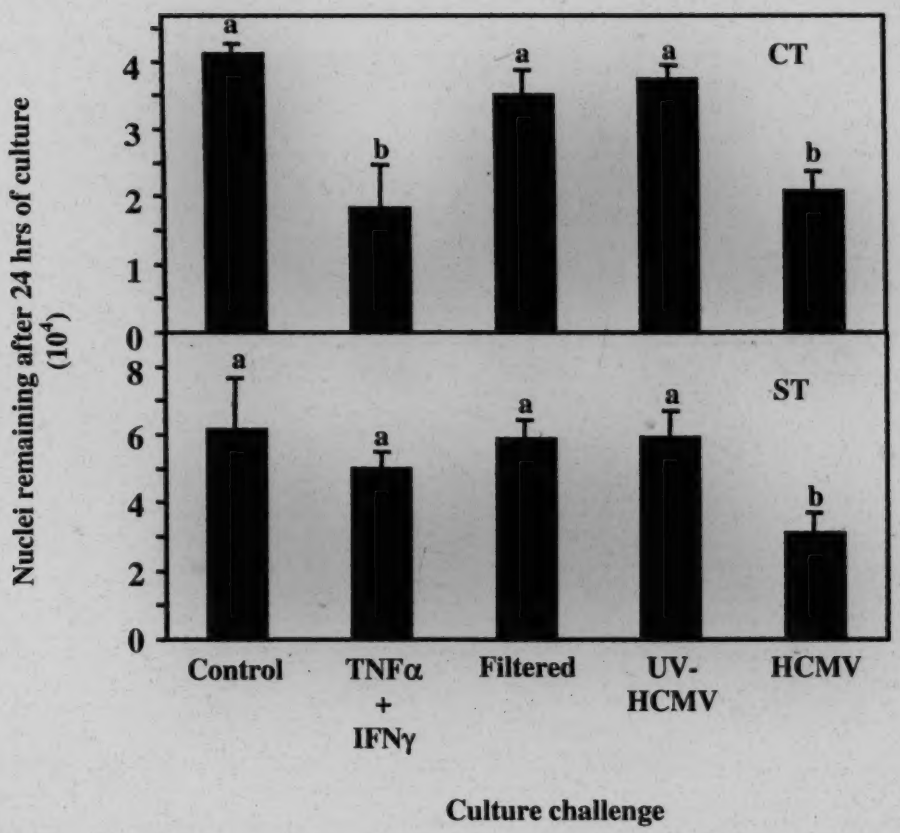

Figure 3.2: Nuclei loss from trophoblast cultures after HCMV infection.

CT and ST cultures were given medium alone (Control), treated with TNFα/IFNγ, or challenged with filtered, UV-inactivated or active HCMV strain AD169 virus preparations at an MOI of 10. After 24 hrs, the number of nuclei remaining in CT (upper panel) and ST (lower panel) cultures was determined by DAPI staining. Depicted is the mean ± SD of three independent experiments (each with duplicate microwells) using cells from two different placentae. Experimental groups within each panel labeled with different letters (a or b) are significantly different ($P<0.05$).

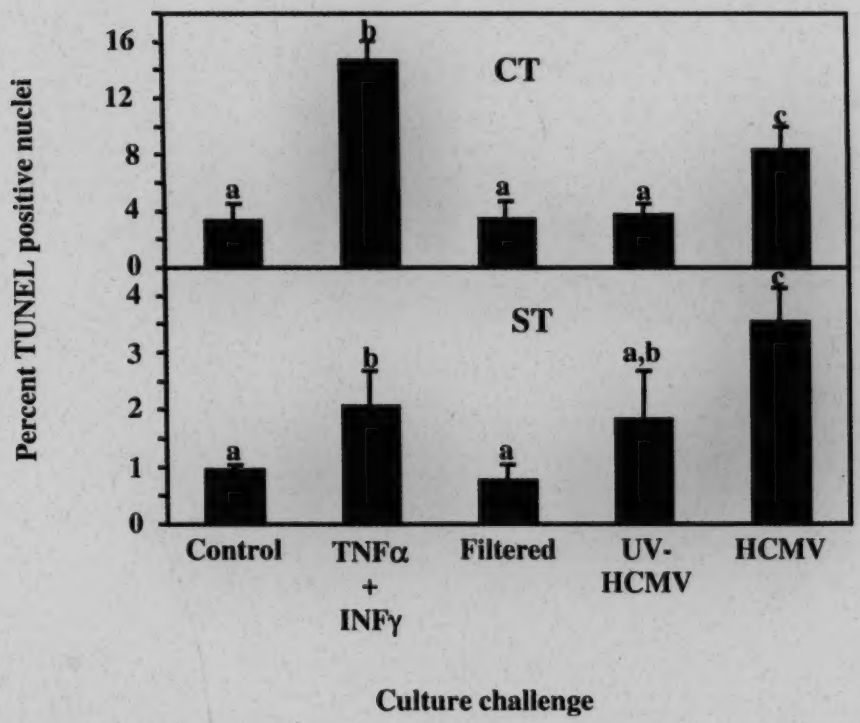

Figure 3.3: HCMV-induced trophoblast apoptosis 24 hrs after challenge.

CT and ST cultures were given medium alone (Control), treated with TNFα/IFNγ, or challenged with filtered, UV-inactivated or active HCMV strain AD169 virus preparations at an MOI of ten. After 24 hrs, the fraction of apoptotic nuclei in CT (upper panel) and ST cultures (lower panel) was determined by TUNEL analysis. Depicted is the mean ± SD of three independent experiments (the same as depicted in Fig 2). Experimental groups within each panel labeled with different letters (a, b or c) are statistically different ($P<0.05$).

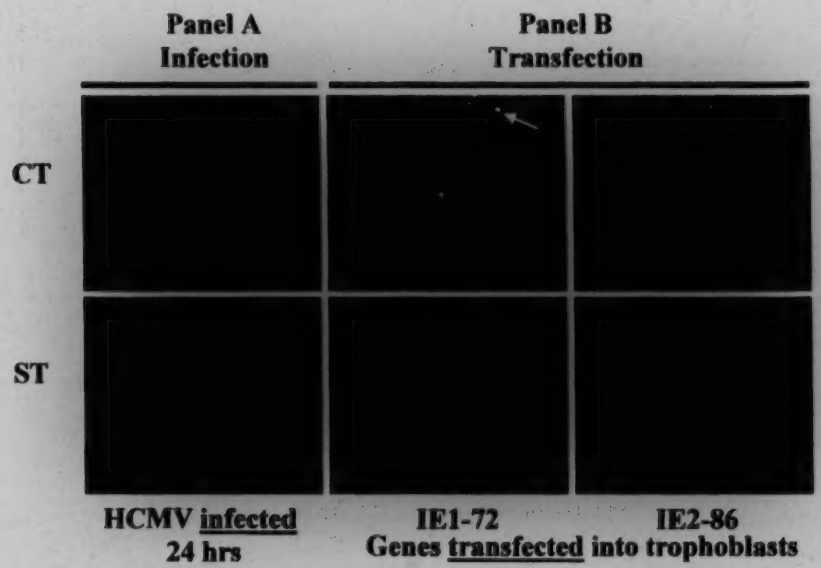

Figure 3.4: Relationship of trophoblasts expressing nuclear HCMV-IE antigen and those undergoing apoptosis during infection by HCMV (Panel A) and transfection (Panel B) by HCMV IE genes.

CT and ST trophoblasts were challenged with HCMV strain AD169 at an MOI of ten (panel A) or transfected with HCMV IE1-72 or IE2-86 expression plasmids (panel B) as described in the Methods and Materials. After 24 hrs, cultures were fixed, stained for nuclear DNA nicking (TUNEL) and immuno-stained for nuclear expression of HCMV-IE antigen and visualized by fluorescence [IE with Alexa Fluor 546, (red); TUNEL with Alexa Fluor 488 (green)] as described in the Methods. Double-labeling for IE proteins and TUNEL gives a yellow color (see arrow in CT panel B). This experiment was carried out three times with cells from two different placentae with the same results.

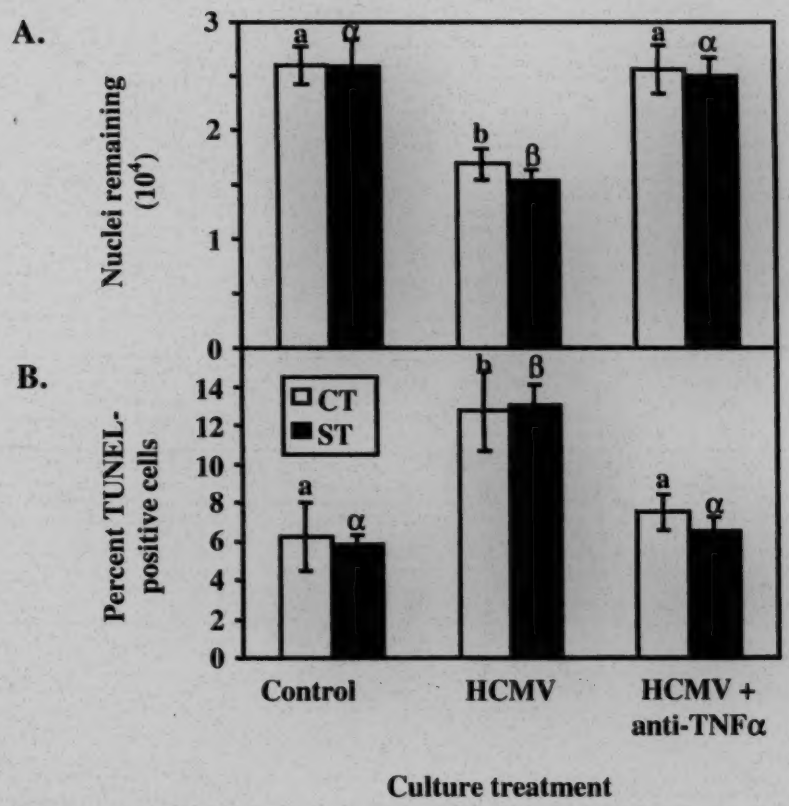

Figure 3.5: The effect of neutralizing antibody to TNFα on HCMV-induced trophoblast cell loss (panel A) and apoptosis (panel B).

CT (open bars) and ST (filled bars) cultures were challenged with HCMV strain AD169 at an MOI of ten and cultured for 24 hrs in the presence and absence of 20 μg/ml TNFα antibody, as described in the Methods. Nuclei remaining in culture and percent apoptotic nuclei were quantitated as detailed in the Methods and Materials. Depicted are the averages ± SD of pooled results from two independent experiments, each containing two replicate wells. Experimental groups within each panel and cell type labeled with different letters (a or b for CT, α or β for ST) are significantly ($P<0.05$) different.

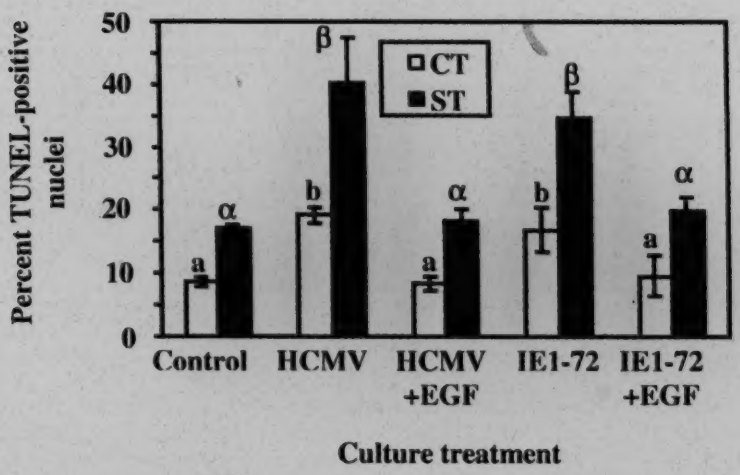

Figure 3.6: The effect of EGF on apoptosis induced by HCMV infection and by transfected HCMV IE genes in CT and ST cultures.

CT and ST cultures were prepared and challenged with HCMV, or transfected with HCMV-IE1-72 or IE2-86 expression plasmids. Apoptosis was assessed by TUNEL analysis 24 hrs after challenge as described in the Methods and Materials. Depicted are the averages ± SD of triplicate samples from a three independent experiments using cells from two different placentae. Experimental groups within cell type labeled with different letters (a or b for CT, α or β for ST) are significantly ($P<0.05$) different.

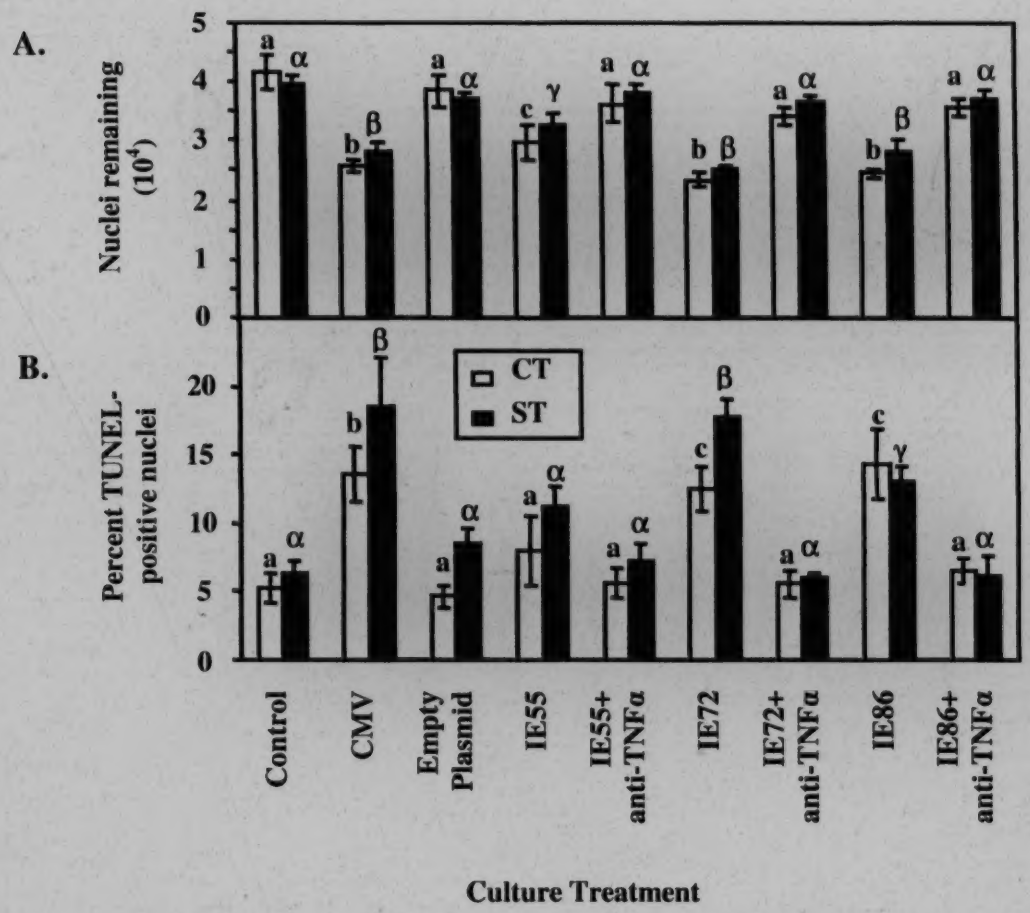

Figure 3.7: The effect of neutralizing antibody to TNFα on IE gene-transfected trophoblast-induced cell loss (upper panel) and apoptosis (lower panel).

CT (open bars) and ST (filled bars) cultures were infected with HCMV strain AD169 at an MOI of 10 or transfected with empty vector, IE2-55, IE1-72 and IE2-86 expressing plasmids and incubated with or without anti-TNFα antibody as indicated. Nuclei remaining and percent TUNEL positive nuclei were determined after 24 hrs culture, as described in the Methods and Materials. Depicted are the averages ± SD for two independent experiments with cells from different placentae. Experimental groups within each panel and cell type labeled with different letters (a, b or c for CT; α, β or γ for ST) are significantly ($P<0.05$) different.

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CHAPTER 4: UV-INACTIVATED HUMAN CYTOMEGALOVIRUS INDUCES APOPTOSIS IN PLACENTAL SYCYTIOTROPHOBLASTS BY TOLL-LIKE RECEPTOR-2-MEDIATED RELEASE OF TUMOR NECROSIS FACTOR α

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4.1 Introduction

Primary HCMV infection acquired later during pregnancy results in approximately 25% of affected neonates exhibiting IUGR (Istas *et al.* 1995). HCMV infection is a major cause of chronic villitis (Benirschke *et al.* 1974; Schwartz *et al.* 1992; Benirschke and Kaufmann 2000) and is accompanied by focal loss of the villous ST (Benirschke and Kaufmann 2000). Cultured villous trophoblasts can be permissively infected by HCMV (Halwachs-Baumann *et al.* 1998; Hemmings *et al.* 1998) and are susceptible to HCMV-induced, HCMV IE gene mediated apoptosis (Chan *et al.* 2002). ST from infected villous explants or from villi infected *in utero* rarely expressed IE proteins (Fisher *et al.* 2000) but even in the absence of virus replication (a latent infection), HCMV can still induce lesions that localized to the trophoblastic cells covering the placental villi in first trimester human placental explants (Amirhessami-Aghili *et al.* 1987). Therefore, IE proteins may contribute little to ST loss because of their limited expression *in vivo* and if so, it remains unknown how HCMV causes widespread inflammation and ST loss.

TLRs belong to an ancient family of receptor proteins involved in the inflammatory responses to various pathogens (Kurt-Jones *et al.* 2000; Rassa and Ross 2003) and are activated by recognizing PAMPs commonly found on pathogens. TLRs signal through the TIR domain found in the cytoplasmic tail which binds the adaptor protein MyD88. IRAK then binds and becomes phosphorylated allowing association with TRAF6 which activates NF-kB, JNK, p38 and ERK pathways upstream of the secretion of a number of inflammatory cytokines including TNFα and IL-1 (Rassa and Ross 2003).

Binding of HCMV particles to host cell surface leads to the rapid activation of transcriptions factors NF-κB and SP-1 (Boldogh *et al.* 1993; Yurochko *et al.* 1995;

Yurochko *et al.* 1997) which could be mimicked by HCMV glycoproteins gB or gH alone (Yurochko *et al.* 1997). UV-HCMV also induce the expression of inflammatory cytokines (Browne *et al.* 2001). HCMV viruses activate an inflammatory response in a TLR2 and CD14-dependent manner such that cells lacking either receptor were significantly impaired in triggering an inflammatory response or activating the NF-κB pathway (Compton *et al.* 2003). Soluble gB also induces secretion of inflammatory cytokines in a TLR2-dependent manner (Boehme and Compton 2004) and appears to directly interact with a heterodimer of TLR2 and TLR1 (Rassa and Ross 2003). Term (Holmlund *et al.* 2002) or first trimester (Abrahams *et al.* 2004) trophoblasts express TLR2 which mediates apoptosis (Abrahams *et al.* 2004).

We therefore hypothesize that UV-HCMV interacts with TLR2 on term ST to induce TNFα release and trophoblast apoptosis. We find that UV-HCMV increases apoptosis in ST cultures, leads to an increase in TNFα gene transcription and translation in a TLR2 dependent manner and that antibody to TLR2 blocks the UV-HCMV increase in apoptosis. This study suggests for the first time that virus gene transcription is not required for trophoblast loss in HCMV-caused villitis.

4.2 Materials and Methods

4.2.1 Cells

See Chapter 3.2.1

4.2.2 Virus preparation, culture Challenge, and assessment of infection

HCMV laboratory strain AD169 and clinical strain Kp7 purified as previously described (Taylor and Cooper 1990). The supernatant of HCMV-infected HEL monolayers was collected from 600 ml tissue culture flasks and centrifuged at 4000 x *g* for 15 to remove cellular debris. The virus was pelleted from the supernatant by centrifugation at 19,000 rpm in a Beckman (Fullerton, CA) SW28 rotor for 1 hr through a 2-ml layer of 20% (wt/vol) D-sorbitol. The pellets were resuspended in PBS and then layered onto a 20 to 70% (wt/vol) linear D-sorbitol step gradient and centrifuged at 85,000 x *g* for 1 hr. A band of viral particles was collected at the 50 to 60% interface and diluted in 4 volumes of PBS. The virus was pelleted by centrifugation at 85,000 x *g* for 1 hr.

The pellets were resuspended in 2% FBS-IMDM. Aliquots were frozen at -70°C and stored in liquid nitrogen until use.

Viral titers were determined by inoculating confluent HEL cultures in 96-well plates with dilutions of each virus preparation in 2% FBS-IMDM. The plates were then centrifuged for 45 min at 2,500 rpm in a GCL-2 Sorvall (Guelph, ON, Canada) centrifuge, the wells washed five times with warm IMDM and the plates incubated for a further 18 to 20 hrs in fresh 2% FBS-IMDM. The cultures were fixed in ice-cold methanol and immunohistochemically stained for HCMV IE antigen as described below. Each IE-positive nucleus was equated to an IF of infectious virus, and the titer of virus was determined within a linear dose-response concentration range as IF/ml.

ST cultures were virus challenged five days after plating and then washed once with warm IMDM and challenged in 2% FBS/IMDM for 24 hrs. The virus challenge was at a MOI of ten, calculated by first enumerating, in parallel cultures, the number of nuclei in ST cultures by DAPI staining (see below) and then adding a ten-fold higher IF of virus (or an equal volume of UV-inactivated HCMV). HCMV was inactivated by exposure to UV-light (30 W, Germicidal, distance of 20 cm) on ice for 20 min. UV inactivation was assured by the absence of IE positive nuclei in trophoblast cultures. After 24 hr culture, the cells were washed twice with phosphate-buffered saline (PBS), fixed in ice-cold acetone:methanol (1:1) for 10 min at –20°C, and washed three times with PBS in preparation for immunofluorescence and/or TUNEL (see below). In experiment to neutralize TNFα or inhibit TLR2 signaling, 10 μg/ml of polyclonal anti-human TNFα (Upstate Biotechnology; Lake Placid, NY) or anti-human TLR2 (clone TL2.1; eBioscience, San Diago, CA) was added to the culture 2 hrs before virus challenge, respectively. In experiments in which cycloheximide (CHX) was used, 10 μg/ml was added to the cells 1 hr prior to virus challenge and continued during infection. Following 24 hrs incubation, cells were washed with PBS three times, fixed and analyzed as described below.

4.2.3 Immunofluorescence staining

Two-color fluorescence analysis was carried out to examine nuclei expressing HCMV IE proteins. After acetone:methanol fixation and PBS washing, non-specific

binding sites were blocked with non-immune goat serum (Zymed Laboratories; Markham, CA) and primary antibody to HCMV IE (detecting p72; Specialty Diagnostics, Dupont) or its IgG1 isotype control (Dako Corporation, Carpinteria, CA) added and incubated at 20°C for 1 hr. The primary antibody was then removed, the cells washed five times with PBS and were incubated with 50 µl per well of 1 µg/ml Alexa Fluor 488 anti-mouse IgG conjugate (Molecular Probes, Eugene, OR) for 1 hr at 20°C. The cells were then washed five times with PBS. To visualize all nuclei 100 µl of 1.4 µg/ml DAPI was added to each well and allowed to sit for 10 min at 20°C. Cells were then washed with PBS five times and visualized with a fluorescence microscope (see below). The total number of nuclei (DAPI, blue), IE positive (Alexa Fluor 488, green) and were determined per well by digital analysis as described below.

Two-colour fluorescence analysis was carried out to determine the fraction of nuclei containing nicked double-stranded DNA (a marker of apoptosis). After acetone:methanol fixation, and PBS washing, the fraction of nuclei with nicked DNA was determined by TUNEL (Gavrieli *et al.* 1992) as previously described (Yui *et al.* 1994). After the reaction was terminated by adding double-strength 300 mM sodium chloride plus 30 mM sodium citrate (2x SSC), the cells were washed three times with double distilled water and non-specific binding sites blocked with 3% skim milk/0.5% Tween 20/PBS and nonspecific binding sites blocked with non-immune goat serum (Zymed Laboratories) for 30 min. Following blocking period, cells were washed five times with PBS and 50 µl per well of streptavidin Alexa Fluor 488 conjugate (Molecular Probes) diluted in PBS to 1 µg/ml added and incubated for 1 hr at 20°C. The cells were then washed five times with PBS. To visualize all nuclei 100 µl of 1.4 µg/ml DAPI (Molecular Probes) was added to each well and allowed to sit for 10 min at 20°C. Cells were then washed with PBS five times and visualized with a fluorescence microscope (see below). The total number of nuclei (DAPI, blue) and TUNEL positive (Alexa Fluor 488, green) were determined per well by digital analysis as described below.

4.2.4 Digital photography and analysis

For analysis of IE protein expression, apoptosis and the number of adherent pUL32-EGFP-HCMV viral particles bound to the ST surface, fluorescent images were

obtained with an Olympus IX2-UCB motorized inverted research microscope equipped with a Lambda DG-4 high speed filter changer and a Cascade 16 bit digital monochrome camera (Olympus; Melville, NY). Digital images of each well were obtained with DAPI (blue), rhodamine (red) and FITC (green) filters. We used Slidebook 3.0 (Carsen; Markham, ON, Canada) as capture software and Image Pro-Plus (Media Cybernetics; Del Mar, CA) for analysis.

4.2.5 Western blot analysis of IE protein expression

Trophoblast were seeded in 6-well plates and infected with AD169 as described above. The cells were then harvested with lysis buffer (25 mM Tris–HCl, 150 mM NaCl, 0.1 per cent Triton X-100, 5 mM EDTA [pH 7.4], phosphatase inhibitor cocktail and protease inhibitor cocktail [Sigma]) by scrapping and incubating 20-25 min on ice. The lysates were cleared by centrifugation at 4°C (2 min, 16000×*g*) and stored at -20°C until analyzed. Sample protein concentrations were determined in duplicate with Micro BCA Reagent (PIERCE; Rockford, IL) using a serum albumin standard. Sample protein (15–20 μg) was solubilized in 4x sample buffer (Sigma) by boiling for 5 min and stored until electrophoresis. Sample protein concentrations were determined in duplicate with Micro BCA Reagent (PIERCE) using a serum albumin stock standard. Equal amounts of total cellular protein from each sample were separated using SDS-(7.5%) PAGE followed by immunoblotting. IE72 and β-actin were detected with monoclonal anti-IE72 antibody (Chemicon; Temecula, CA) and anti-β-actin antibody (Sigma), respectively. The blots were incubated overnight at 4°C with specific antibodies, washed twice with the blocking and washing solution, incubated with 1:2000 diluted horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology; Santa Cruz, CA) for 1-2 hrs at 20°C, washed extensively and developed using enhanced chemiluminescence (Amersham Pharmacia Biotech; Buckinghampshire, UK) on X-ray films.

4.2.6 Immunohistochemical staining and reverse-transcriptase polymerase chain reaction (RT-PCR) analysis of TLR-2 expression

ST cultures were washed twice with PBS, fixed with 4% phosphate buffered paraformaldehyde for 10 min at 20°C, and washed three times with PBS again.

Trophoblasts were then incubated with 10% goat serum (Zymed) to block nonspecific binding. Primary antibody against human TLR2, ICAM-1 or its isotype control, IgG1 (Dako Corporation; Carpinteria, CA), were then added and allowed to incubate for 60 min at 20°C. After thorough washing with PBS, secondary antibody (biotinylated goat anti-mouse IgG) and streptavidin-peroxidase conjugate (streptavidin-biotin system, Histostain-SP kit; Zymed) were added according to the manufacturer's instructions.

For RT-PCR analysis of TLR2 gene expression, total RNA from ST was extracted with Trizol (GIBCO) as described by the manufacturer. Total RNA extracted was resuspended in sterilized distilled water. RNA concentration was determined by measuring absorbance at 260 nm. Reverse transcription was performed on 2 μg of total RNA using the TaqMan reverse transcription (RT) kit (Invitrogen), containing 1 X RT buffer (Invitrogen RT buffer), 5.5 mM $MgCl_2$, 500 μM deoxyNTP, 2.5 μM random primers, 0.4 U/μl RNase inhibitor, and 1.25 U/μl reverse transcriptase in a total volume of 40 μl. Control mixtures were prepared for each RNA sample containing no reverse transcriptase and a no-template control was also included. cDNAs were amplified using 2 U of Taq polymerase (Invitrogen) in a 30 μl reaction volume containing 10 μM of TLR2 specific primers, 10 μM of each deoxynucleoside triphosphate, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, and 1.5 mM $MgCl_2$. Primer pairs specific for TLR2, sense, 5'-GCCAAAGTCTTGATTGATTGG-3'; antisense, 5'-TTGAAGTTCTCCAGCTCCTG-3' (Varadaradjalou *et al.* 2003), were purchased from DNA Core Services Laboratory (University of Alberta, Edmonton, Canada). The cycling parameters were as follows: initial denaturation at 95°C for 5 min then 35 cycles at 95°C for 20 s, 54°C for 45 s and 72°C for 60 s. Amplification of glyseraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control. The PCR products were electrophoresed on a 1.5% agarose gel and detected with ethidium bromide

4.2.7 Real-time RT-PCR analysis of TNFα mRNA expression

Total RNA was extracted from ST and quantitated as described above. Total RNA (1000 ng) was reverse transcribed using Supercript II reverse transcriptase enzyme (Invitrogen) in a volume of 20 μl using the protocol supplied by the manufacturer. To exclude contamination of cDNA samples with genomic DNA, random samples had

corresponding reactions where Superscript II was replaced with water (No RT controls). The primers and probe specific for human TNFα and GAPDH were purchased from Applied Biosystems (Foster City, CA). The PCR mix consisted of 2 μl cDNA from the RT reaction, Taqman universal PCR master mix containing: AmpliTaq Gold DNA polymerase, AmpErase UNG, dNTPs with dUTP (Applied Biosystems), the mixture of primers and TaqMan probe for human TNFα, and RNAase-free water. The PCR mix was initially heated to 50°C for 2 min and then at 95°C for 10 min for optimal Amperase UNG enzyme activity and to activate AmpliTaq Gold DNA polymerase, followed by 40 cycles of 90°C for 15 s, 60°C for 60 s.

TNFα mRNA measurements were normalized to GAPDH mRNA. Standard curves for both TNFα and GAPDH were generated by serial dilutions of pooled cDNA samples. The amplification efficiency for each primer set was determined by converting the slope of the standard curve using the algorithm $E = 10^{-1/slope}$. For each gene, the mean threshold cycle was corrected for the efficiency of the reaction and was expressed relative to a control sample for each experiment (Pfaffl 2001). TNFα levels were then expressed relative to GAPDH levels using the following formula: $Ratio = \frac{E_{FP}^{\Delta Ct(Control-Sample)}}{E_{GAPDH}^{\Delta Ct(Control-Sample)}}$. The mean of each treatment group was determined. Results for all mRNA measurements were analyzed by either a two way analysis of variance. Significance was achieved at $p<0.05$.

4.2.8 Cytokine assays

Trophoblast were plated in 96-well dishes as described above and incubated with media or challenged with UV-HCMV or HCMV at a MOI of 10. Control cultures were stimulate with zymosan (Sigma; 10 μg/ml) or LPS (Escherichiacoli 0111:B4; Sigma; 100 ng/ml). Prior to use, the LPS was repurified by phenol extraction to remove contaminating lipopeptides, as previously described (Hirschfeld *et al.* 2000). The cultures were incubated at 37°C for 19 hrs and then the culture supernatant harvested. Levels of TNFα and IL-8 secretion were determined by human cytokine-specific enzyme-linked immunosorbent assays (ELISAs) kits (Anogen; Missisauga, ON, Canada) according to manufacturer's directions.

4.2.9 Statistical analysis

See Chapter 3.2.7

4.3 Results

4.3.1 UV-inactivated HCMV induces ST apoptosis

ST cultures were challenged with AD169 or UV-HCMV at a MOI of 10. Following 24 hrs of challenge, trophoblasts were fixed and the frequency of apoptosis determined by TUNEL. As we have previously observed, infection with strain AD169 HCMV resulted in a 3-fold increase in apoptosis from ~6% to ~18% (Fig 4.1A) (Chan *et al.* 2002). UV-HCMV strains AD169 and a clinical variant Kp7 were able to induce a lesser, but significant, amount of ST apoptosis (Fig 4.1A, B). Inactivation of the virus by UV was confirmed by the absence of IE expression (Fig 4.1C and 4.4C, lane 2).

4.3.2 Syncytiotrophoblast express TLR2

We have previously shown that HCMV was able to induce trophoblast apoptosis and that this effect could be mimicked by expressing individual IE proteins (Chan *et al.* 2002). IE gene transfection stimulated the secretion of TNFα (Chan *et al.* 2002; Chan *et al.* 2004) and induced apoptosis in neighboring non-transfected cells (Chan *et al.* 2002). Activation of TLR2 can mediate the release of TNFα in response to Mycobacterium tuberculosis (Underhill *et al.* 1999) and Compton *et al.* have demonstrated that HCMV virus particles can initiate an inflammatory response via TLR2 (Compton *et al.* 2003). Thus, we asked whether cultured trophoblasts express TLR2. RT-PCR indicated expression of TLR2 mRNA in ST cultures (Fig 4.2A) and immunohistochemical staining confirmed protein expression on the cell surface (Fig 4.2B).

4.3.3 UV-inactivated HCMV stimulates the secretion of TNFα via TLR2

Next, we determined if UV-HCMV could activate TLR2 to induce TNFα secretion from ST cultures. ST cultures treated with zymosan, a relatively specific ligand of TLR2 (Underhill 2003), resulted in an increase in TNFα secretion (Fig 4.3A). Cells challenged with UV-HCMV or HCMV also showed higher levels of TNFα in the supernatant. Neutralizing anti-TLR2 antibody blocked zymosan and UV-HCMV-

induced secretion of TNFα and significantly reduced the levels induced by HCMV. Since LPS [known to initiate an inflammatory response via TLR4 (Chow *et al.* 1999)] did not stimulate the release of measurable TNFα (Fig 4.3A), we examined the secretion of IL-8 to ensure the specificity of TLR2 antibody (Fig 4.3A). As expected, TLR2 antibody blocked zymosan- but not LPS-stimulated release of IL-8. These results suggest that inhibition of TLR2 blocks UV-HCMV induced secretion of TNFα.

Using real time RT-PCR, we show that virus binding and perhaps entry is necessary for stimulating transcription of or stabilizing TNFα mRNA. UV-HCMV challenged ST cultures showed an approximate 4-fold increase in TNFα mRNA and the presence of TLR2 antibody reduced mRNA levels to that in the control cultures (Fig 4.3B). Infection by HCMV resulted in a 6-fold increase in transcript levels and although pretreatment of anti-TLR2 significantly reduced the amount of mRNA, it did not completely reduce the levels of TNFα transcript. The presence of cycloheximide during UV-HCMV challenge could not block the increase in TNFα gene transcripts (Fig 4.3C) indicating that UV-HCMV increased the levels of TNFα mRNA independent of de novo protein synthesis. Similar treatment of HCMV-infected cultures with cyclohexamide reduced the amount of TNFα mRNA to levels found in cultures challenged with UV-HCMV. This finding, taken together with observations that cycloheximide blocks IE72 protein expression (Fig 4.3D, lane 5) and HCMV IE proteins can stimulate production of TNFα (Geist *et al.* 1994; Geist *et al.* 1997; Chan *et al.* 2002), suggest that HCMV IE gene products are responsible for about 1/3 of the increase in TNFα levels during a 24 hr exposure to HCMV.

4.3.4 TNFα and TLR2 mediate UV-HCMV induced syncytiotrophoblast apoptosis.

The above results indicate that binding of HCMV activates TLR2 to signal the production and secretion of TNFα. We next asked if the release of TNFα can account for the death stimulated by UV-HCMV. Challenge with UV-HCMV doubled the apoptosis frequency of trophoblasts (Fig 4.1, Fig 4.4). Neutralizing antibody to TNFα inhibited UV-HCMV induced apoptosis to the medium control (Fig 4.4A). Since TNFα is responsible for the death induced by UV-HCMV, blocking TLR2 signaling should block death as well. Indeed, we find that the presence of TLR2 antibody abrogated apoptosis

induced by UV-HCMV (Fig 4.4B). However, TLR2 antibody inhibition of death was not seen in cultures infected with HCMV. Although anti-TLR2 slightly reduced HCMV-induced apoptosis, the reduction was not significant. This result is in agreement with our finding that TLR2 antibody does not completely block IE production (Fig 4.3D) and with previous observations that HCMV IE genes stimulate the secretion of TNFα (Geist *et al.* 1994; Geist *et al.* 1997; Chan *et al.* 2002).

4.4 Discussion

Placentae isolated from infants diagnosed with congentital HCMV infection often exhibit widespread villous inflammation (chronic villitis) (Garcia *et al.* 1989; Schwartz *et al.* 1992; Sinzger *et al.* 1993). Villitis caused by HCMV is characterized by the focal loss of trophoblast resulting in the formation of lesions and loss of trophoblast barrier function (Garcia *et al.* 1989; Benirschke and Kaufmann 2000). We have previously shown that HCMV infection of primary villous trophoblasts (both CT and ST) in the first 24 hrs of culture resulted in the induction of apoptosis mediated by the release of the cytokine TNFα (Chan *et al.* 2002). Expression of individual HCMV IE proteins (IE1-72, IE2-86 and IE2-55) stimulated apoptosis in a similar fashion suggesting their involvement in the process. In the present study we find that the binding of transcriptionally silent UV-HCMV particles to cell surface TLR2 on ST activates and initiates a signal to secrete sufficient TNFα to induce trophoblast apoptosis. Thus, binding and perhaps internalization of virus particles alone (without initiation of virus gene transcription) can increase TNFα release by the ST to levels dangerous to underlying CT and perhaps surrounding ST. This observation has implications for HCMV infections in general, where a large fraction of virus secreted is transcriptionally inactive (dense bodies) (Sarov and Abady 1975), and more particularly for pregnancy during which latent infections commonly reactivate (Spano *et al.* 2004). The increased virus secretion during pregnancy, including dense bodies, could cause the massive chronic villitis seen in HCMV infected placentae and also lead to possible consequences of such villitis: transmission of the virus across the placenta and intrauterine growth restriction.

The present studies also have major implications for the involvement of TLRs in HCMV replication. HCMV infection leads to the activation of NF-κB (Boldogh *et al.*

1993; Kowalik *et al.* 1993) in a biphasic manner (Yurochko *et al.* 1995). The first tier of induction is insensitive to cycloheximide indicating a receptor-ligand interaction, whereas the second tier of induction was sensitive to cycloheximide and required IE gene expression (Yurochko *et al.* 1995). Activation of NF-κB is crucial to the replication of HCMV: it is required for transactivation of the HCMV IE promoter (DeMeritt *et al.* 2004), HCMV IE proteins are necessary for virus replication (Landolfo *et al.* 2003), IFNα inhibits murine HCMV IE gene expression by down-regulating NF-κB (Prosch *et al.* 1995) and stimulation of the HCMV IE promoter by TNFα is mediated by NF-κB (Gribaudo *et al.* 1995). Virus binding and perhaps uptake with TLR2 leads to the induction of NF-κB (Stuart *et al.* 2005) and blocking TLR2 signaling in ST with TLR2 antibody significantly reduced the amount of IE gene expression without inhibiting virus entry (see Fig 4.3). Although more detailed experiments are required, our data indicates that TLR2 may play an important role in the earliest stages of HCMV replication by upregulating NF-κB.

These studies suggest differences in TLR2 expression and signaling between first trimester and term trophoblasts. Term ST are reported to express TLR2 (Holmlund *et al.* 2002) but first trimester ST are not (Abrahams *et al.* 2004), thereby indicating a maturation state difference in expression. However, the latter authors also reported that TLR2 expressed by first trimester CT cell lines responded to the peptidoglycan PDG by undergoing apoptosis. Since PDG ligation moderately decreased cellular expression of TNFα and activated FADD, caspases 3, 8 and 9, and inactivated XIAP, these authors concluded that TLR2 directly activated the caspase cascade. However, we find that cultured term villous ST express TLR2, which agrees with *in vivo* data (Holmlund *et al.* 2002), but that these cells undergo apoptosis stimulated by UV-HCMV (and mediated by TLR2) indirectly by upregulating TNFα (Fig 4.3 and 4.4). The two studies cannot be directly compared: Abrahams *et al* used first trimester CT cell lines stimulated with a peptidoglycan contained in a bacteria (PDG) and ours used term ST stimulated by UV-HCMV. However, both involve TLR2 ligation in trophoblasts and Abrahams *et al* did not test whether TNFα intermediated apoptosis.

The studies also indicate response differences mediated by TLR2 in term villous CT and ST stimulated by UV-HCMV. We previously reported that term CT [which

express TLR2 mRNA (Holmlund *et al.* 2002)] do not increase apoptosis in response to UV-HCMV (Chan *et al.* 2002) in contrast to term ST (present chapter). Both differentiation states undergo TNFα-mediated (Garcia-Lloret *et al.* 1996) and HCMV-induced (Chan *et al.* 2002) apoptosis. However, it is not known if UV-HCMV induces TNFα expression in a TLR2-dependent manner in CT as it does in ST. These results indicate the complexity of innate responses to HCMV and suggest that another co-receptor [perhaps TLR1, (Rassa and Ross 2003)] is not present in CT and is involved in mediating the response in term ST.

The role of the villous placenta, specifically the ST which is in direct contact with maternal blood, in vertical transmission of HCMV is unknown. Chronic villitis is often seen in HCMV infected placentae (Garcia *et al.* 1989; Schwartz *et al.* 1992; Sinzger *et al.* 1993) and cultured villous trophoblasts (both CT and ST) can be infected by HCMV (Halwachs-Baumann *et al.* 1998; Hemmings *et al.* 1998). However, tissue sections of villous explants challenged with HCMV for 4 days express IE proteins in the CT and extremely rarely in the ST and chrorionic villi that were infected with HCMV *in utero* show a similar pattern of infection (Fisher *et al.* 2000). These observations suggest a route through the anchoring villi where the infection spreads from the maternal uterine cells to the invasive CTs. Infection would bypass the ST and any infection of the layer would be rare and likely to occur later in the infection process (Fisher *et al.* 2000). Although this model of vertical transmission can account for the lack of productive infection in the ST, it does not completely explain the widespread villitis and loss of trophoblast seen in placentae from congenially infected fetuses. Another possibility is transmission through sites of damage of the villous ST layer. The loss of trophoblast seen in the villous placenta from HCMV infected placenta results in the formation of lesions and loss of ST (Benirschke *et al.* 1974; Garcia *et al.* 1989; Schwartz *et al.* 1992; Sinzger *et al.* 1993). In this study, replication deficient HCMV can stimulate apoptosis by binding to TLR2 leading to secretion of TNFα and trophoblast apoptosis. These results suggest that virus interacting with TLR2 without any translational evidence of infection is sufficient to induce ST damage and thereby allow passage of virus to the underlying cytotrophoblasts and fetal stroma. Thus, it is premature to eliminate a route and, indeed, one route may dominate in early pregnancy and another later.

The turnover of villous trophoblast includes proliferation and differentiation of CT, fusion of underlying CT with the ST, full differentiation of ST and the formation of syncytial knots that pinch off into the maternal circulation (Jones and Fox 1977). It now appears that apoptosis plays a major role in trophoblast turnover (Huppertz *et al.* 1999; Huppertz *et al.* 1999; Burton *et al.* 2003; Black *et al.* 2004). Initiator caspase 8 is required for fusion of CT into ST (Black *et al.* 2004), but the progression of apoptosis does not occur immediately upon fusion of the CT with the ST (Huppertz *et al.* 1998). It is believed that the apoptotic cascade may be initially halted by the large amount of anti-apoptotic Bcl-2 family members present upon fusion (LeBrun *et al.* 1993; Sakuragi *et al.* 1994). The shift from inhibition of apoptosis to execution remains unclear; however, effector caspases 3 and 6 are eventually activated in the ST (Kadyrov *et al.* 2001; Huppertz *et al.* 2003). TNFα, known to initiate the caspase cascade, is produced by the villous placenta (Chen *et al.* 1991) which expresses both p55 and p75 receptors (Yelavarthi and Hunt 1993; Yui *et al.* 1996). However, TNFα also plays a pivotal role in placental development (Hunt *et al.* 1996). Interaction of the villous ST with HCMV would increase TNFα levels and alter the normal rate of trophoblast turnover by increasing ST aging into syncytial knots. It would also compromise maintenance of the ST by inducing apoptosis in the underlying CTs. Over expression of TNFα (Miller *et al.* 1996), increased villous trophoblast apoptosis (Smith *et al.* 1997) and enhanced syncytial knot formation (van der Veen and Fox 1983) are characteristics congruent with placental pathology associated with IUGR.

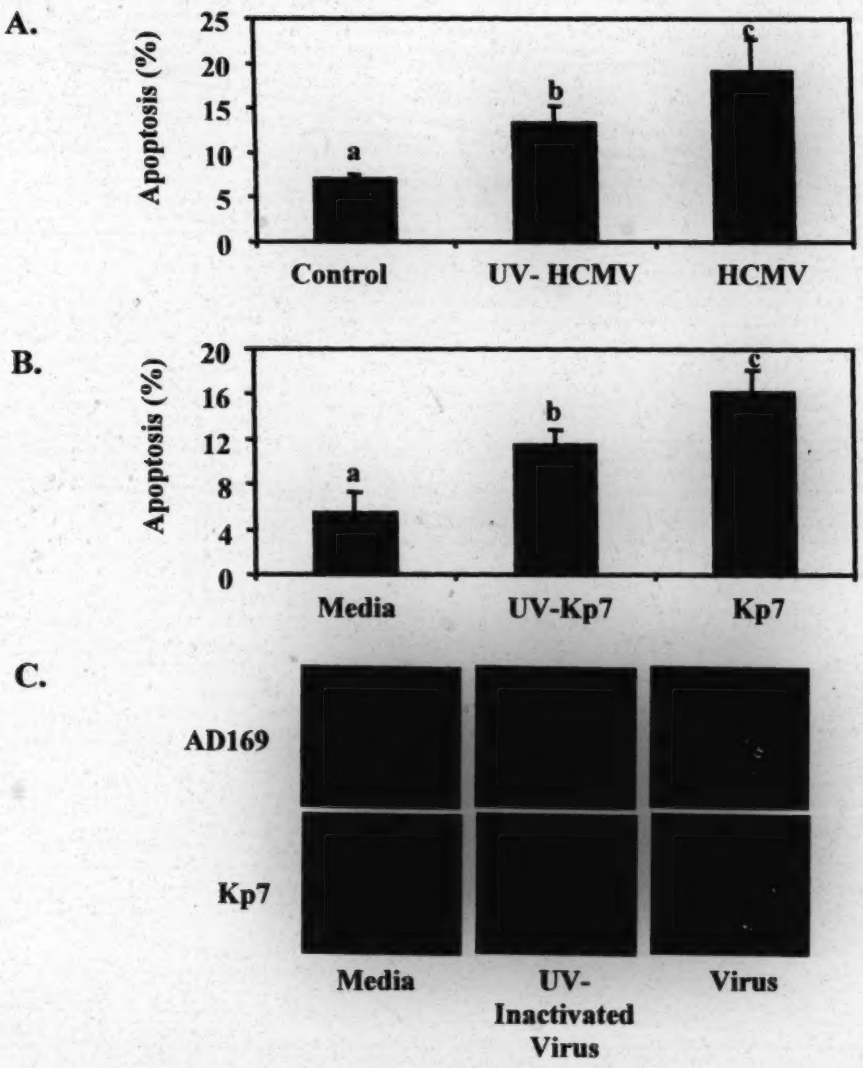

Figure 4.1: UV-inactivated HCMV induced apoptosis 24 hours after challenge.

ST cultures were treated with media, challenged with UV-HCMV or infected with HCMV strain (**A**) AD169 or (**B**) Kp7 at a MOI of 10. After 24 hrs, immediate early antigen IE1-72 expression (**C**) or the frequency of apoptotic nuclei (**A and B**) was determined as described in the Materials and Methods. Depicted is the mean $\pm$ SD of pooled results from six independent experiments using cells from three different placentae. Experiment groups within each panel labeled with different letters (a, b or c) are statistically different ($P<0.05$).

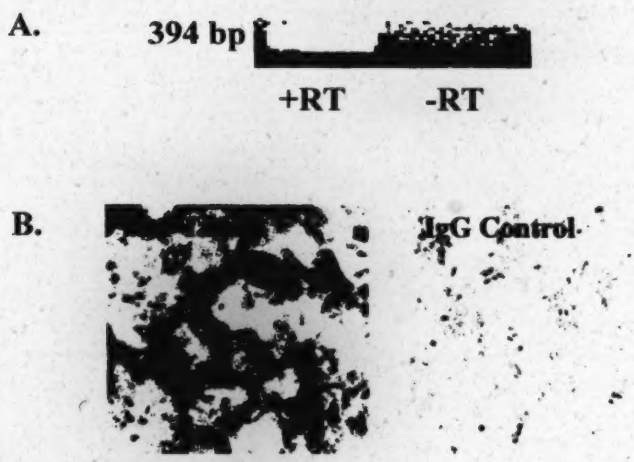

Figure 4.2: ST cultures express TLR2.

Purified villous CT were differentiated in ST cultures in the presence of EGF for 5 days. **(A)** RNA was harvested at day 5 and steady-state TLR2 mRNA levels were analyzed by RT-PCR. Lane 1 = plus reverse transcriptase; lane 2 = minus reverse transcriptase. This gel is a representation of three independent experiments carried out with cells from three different placentae. **(B)** ST cultures were fixed with paraformaldehyde at day 5 and cell surface TLR2 protein expression determined by immunohistochemistry using anti-TLR antibody (5 μg/ml) and IgG negative control. Microphotographs are representative of three independent experiments carried out with cells from two different placentae.

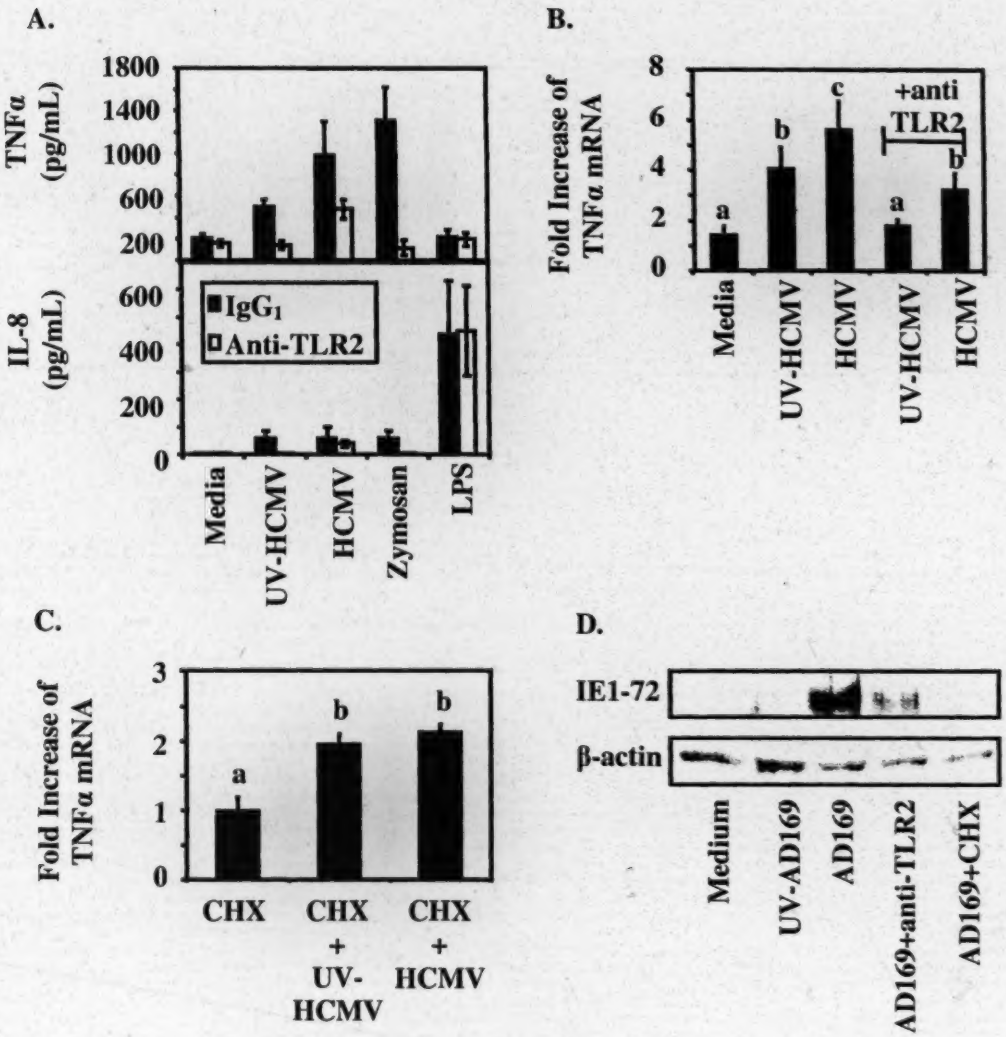
A.
TNFα (pg/mL)
1800
1400
1000
600
200
IL-8 (pg/mL)
600
400
200
0
IgG1
Anti-TLR2
Media
UV-HCMV
HCMV
Zymosan
LPS
B.
Fold Increase of TNFα mRNA
8
6
4
2
0
+anti TLR2
a
b
c
a
b
Media
UV-HCMV
HCMV
UV-HCMV
HCMV
C.
Fold Increase of TNFα mRNA
3
2
1
0
a
b
b
CHX
CHX + UV-HCMV
CHX + HCMV
D.
IE1-72
β-actin
Medium
UV-AD169
AD169
AD169+anti-TLR2
AD169+CHX

Figure 4.3: UV-inactivated HCMV induced production of TNFα.

(**A**) ST cultures were incubated in the presence or absence of anti-TLR2 antibody (10 μg/ml) with no stimulus, UV-HCMV, HCMV strain AD169, zymosan (10 μg/ml; TLR2 ligand), or phenol reextracted LPS (10 ng/ml; TLR2 ligand). The supernatants were collected 19 hrs later, and TNFα and IL-8 levels determined by ELISA. Total RNA was harvested from ST cultures following 24 hr treatment (**B**) with no stimulus, UV-HCMV or HCMV in the presence or absence of anti-TLR2 antibody (10 μg/ml) and (**C**) no stimulus, UV-HCMV or HCMV in the presence of (10 μg/ml) CHX. Steady-state TNFα mRNA levels were analyzed by real-time RT-PCR. Depicted is the mean ± SD of pooled results from three independent experiments using cells from two different placentae. Experiment groups within each panel labeled with different letters (a, b or c) are statistically different ($P<0.05$). (**D**) ST cultures were treated in media, challenged with UV-inactivated AD169, or infected with AD169 in the presence or absence of neutralizing antibody to TLR2 or CHX. Following 24 hrs of incubation, cells were harvested and IE1-72 expression examined using western blot analysis. Western blot is a representation of three independent experiments done using cells from two different placentae.

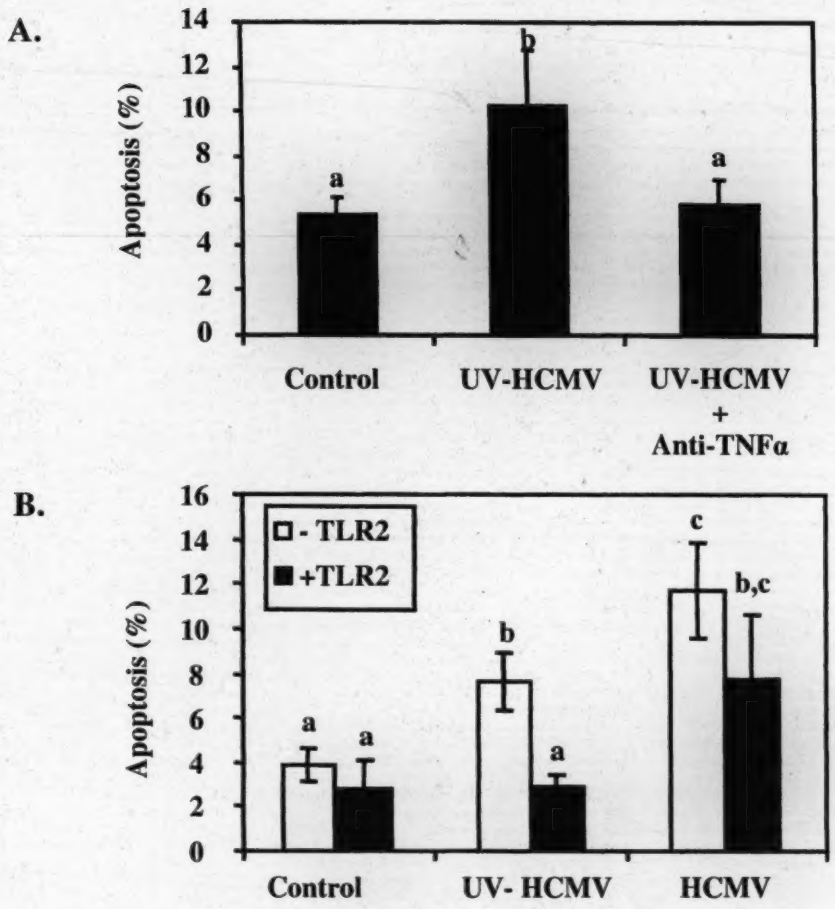

Figure 4.5: The effect of neutralizing antibody to TNFα (A) and TLR2 (B) on UV-HCMV and HCMV-induced ST apoptosis.

ST cultures were treated with no stimulus or challenged with UV-HCMV strain AD169 or HCMV strain AD169 in the presence or absence of antibodies (10 μg/ml) for 24 hours. The fraction of apoptotic nuclei was determined by TUNEL analysis. Depicted is the mean ± SD of pooled results from three independent experiments using cells from two different placentae. Experimental groups within each panel labeled with different letters (a or b) are statistically different ($P<0.05$).

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CHAPTER 5: HUMAN CYTOMEGALOVIRUS-INDUCED UPREGULATION OF INTERCELLULAR CELL ADHESION MOLECULE-1 ON VILLOUS SYNCYTIOTROPHOBLASTS

A version of this chapter has been published. Chan, Stinski and Guilbert. Biol Reprod 2004; 71:797-803.

5.1 Introduction

HCMV is often found in placentae with villitis, inflammation of the placenta (Garcia *et al.* 1989; Sinzger *et al.* 1993; Bernstein and Divon 1997; Benirschke and Kaufmann 2000), which is accompanied by a focal loss of the trophoblast (Benirschke and Kaufmann 2000). The major infiltrating leukocytes in villitis associated with HCMV or with villitis of unknown etiology are mononuclear phagocytes (Altemani 1992; Greco *et al.* 1992) suggesting these to be involved in the disease. Monocyte adhesion to ST cultures *in vitro* is mediated by ICAM-1, which can be stimulated by the inflammatory cytokines IL1β, TNFα and IFNγ (Xiao *et al.* 1997). Such adhesion leads to TNFα-mediated apoptosis (Garcia-Lloret *et al.* 2000) suggesting that villous ST upregulation of ICAM-1 is an early step in villitis.

ICAM-1 is a ~100 kDa membrane glycoprotein, the expression of which is associated with tight immune cell binding to the vasculature (Jutila 1992). It mediates cellular interactions by binding to its receptors on leukocytes, leukocyte function-associated molecule (LFA)-1 and the C3 complement receptor (MAC-1) (Marlin and Springer 1987; Dustin *et al.* 1988), although on trophoblasts ICAM-1 appears to mediate monocyte binding solely through LFA-1 (Xiao *et al.* 1997). ICAM-1 is expressed at relatively low levels on the villous trophoblast unless stimulated by an infection such as *plasmodium falciparum* (Xiao *et al.* 1997; Gaffuri *et al.* 1998; Sugiyama *et al.* 2001). In a number of endothelial and epithelial cell lines, HCMV infection results in the increased expression of ICAM-1 (Grundy and Downes 1993; Craigen and Grundy 1996; Watanabe *et al.* 1996; Shahgasempour *et al.* 1997; Burns *et al.* 1999; Dengler *et al.* 2000). However, nothing is known of the effect of HCMV infection on villous trophoblast expression of ICAM-1.

Using highly purified (>99.99%) CT differentiated into ST cultures, we here show that HCMV-1 infection (or expression of IE-1-72, IE2-86 and IE2-55 genes) upregulates ICAM-1 expression. Interestingly, ICAM-1 expression is stimulated by HCMV in a paracrine fashion (on non-infected cells) by TNFα and to a lesser extent by IL1β but its expression in infected cells is induced in a manner independent of these two cytokines.

5.2 Methods and Materials

5.2.1 Cells

See Chapter 3.2.1.

5.2.2 Virus preparation, culture challenge, and assessment of infection

HCMV laboratory strain AD169 and a GFP expressing HCMV recombinant strain RVdlMwt-GFP, henceforth termed gfp-CMV (Isomura and Stinski 2003), were passaged on confluent HEL cells in 2% FBS-MEM as previously described (Hemmings *et al.* 1998). Infectious virus was recovered by freezing and thawing the cultures three times. The lysate was passed through 0.45-μm-pore-size filters (MILLEX-HV; Millipore Products Division, Bedford, MA) and stored in liquid nitrogen until use. Virus titers were determined by inoculating confluent HEL cultures in 96-well plates with dilutions of each virus in serum-free MEM. The plates were then centrifuged for 45 min at 2,500 rpm in a GCL-2 Sorvall centrifuge, the wells were washed five times with warm MEM, and the plates were incubated for a further 18 to 20 hrs in fresh 2% FBS-MEM. The cultures were fixed in ice-cold methanol and immunohistochemically stained for HCMV IE antigen as described below. Each IE-positive nucleus is equated to an IF of virus, and the titer of virus was determined within a linear dose-response concentration range as IF/milliliter.

UV inactivation of HCMV was by exposure to UV-light (30W Germicidal; samples were exposed at a distance of 20 cm from the UV source) on ice for 20 min. Virus-free supernatant was obtained by filtering HCMV batches through a 0.1-μm-pore-size syringe top filter (Millipore). UV inactivation and complete filtration was assured by the absence of IE-positive nuclei in trophoblasts cultures.

Infections were carried out at a MOI (the ratio of inoculating virus IF to the number of nuclei in the culture to be infected) of 10 in serum-free IMDM for 2 hrs at 37°C in 5% CO_2. The number of trophoblasts present in microwells was determined from parallel cultures by enumerating the number of nuclei in CT and ST cultures by DAPI staining (see below). A 10-fold higher IF of virus (or an equal volume of UV-inactivated or virus-free supernatant from the same preparation) was then added. Cultures were infected with AD169, washed twice with PBS and fixed with 4% paraformaldehyde for 10 min at room temperature. Trophoblasts were then washed three times with PBS in preparation for immunofluorescence (see below). Cultures infected with gfp-CMV were washed five times on day 1 with warm 2% FBS IMDM. Next, 10% FBS IMDM containing EGF was added and changed at day 3. The culture was continued to day 4 (GFP expression can be seen at this time) and then fixed with 4% paraformaldehyde.

5.2.3 Plasmids and transient transfections

See Chapter 3.2.3.

5.2.4 Immunofluorescence staining

ST cultures were challenged with virus-free supernatant, UV-inactivated virus, AD169 or gfp-CMV as described above. Following incubation, cells were washed twice with PBS, fixed with 4% phosphate buffered paraformaldehyde for 10 min at room temperature, and washed three times with PBS again. Trophoblasts were then incubated with 10% goat serum (Zymed/Intermedico; Markham, CA) to block nonspecific binding. Primary antibody against ICAM-1 (1 μg/ml) (ICOS Corporation, Bothell, WA) or its isotype control, IgG1 (Dako Corporation; Carpinteria, CA), were then added and allowed to incubate for 60 min at room temperature. After thorough washing with PBS, Alexa Fluor 546 goat anti-mouse IgG conjugate (Molecular Probes; Eugene, Oregon) was diluted in PBS to 1 μg/ml and 50 μl added to each well for 60 min at room temperature, after which cells were washed 5 times with PBS. Visualization and analysis of immunofluorescence is described below.

Two-color fluorescence analysis was carried out in parallel cultures to determine total nuclei number and the fraction of nuclei expressing HCMV IE proteins. After methanol fixation, and PBS washing, non-specific binding sites were blocked with 3% skim milk/0.5% Tween 20/PBS at room temperature for 30 min. Primary antibodies detecting HCMV IE (detecting p72, p55 and p86; Specialty Diagnostics, Dupont) or its isotype control, IgG (Dako Corporation), were added and incubated at room temperature for 1 hr. The primary antibody was then removed and the cells washed five times with PBS. Next, Alexa Fluor 546 goat anti-mouse IgG1 conjugate (Molecular Probes) was diluted in 3% skim milk/0.5% Tween 20/PBS to 1 μg/ml each and 50 μl added to each well for 60 min at room temperature. The cells were washed with PBS five times and nuclei visualized with 100 μl of 1.4 μg/ml DAPI (Molecular Probes, 10 min, room temperature). The frequency of infection or transfection was determined by number of IE-positive nuclei relative to the total number of nuclei.

5.2.5 Digital photography and analysis

Immunofluorescence was examined using an inverted phase contrast microscope (Model Ds-IRB, Leica; Heerbrugg, Switzerland) equipped for epifluorescence with a 100 W high pressure mercury lamp driven by a Ludl power source (Ludl Electronic Products; Hawthorne, NY). Nuclei and IE-positive nuclei were visualized under a DAPI filter (blue) and a rhodamine filter (red), respectively. ICAM-1 staining was visualized with a rhodamine filter and digital images from each well were taken with a SPOT digital camera (Diagnostic Instruments; St Sterling Height, MI). The mean fluorescent intensity was determined by a digital analysis program, Image-Pro Plus (Media Cybernetics; Del Mar, CA) and normalized to total number of nuclei per image. It should be noted that background fluorescence was corrected for each image by subtracting mean fluorescent intensity from IgG1 stained cultures. GFP expression from the gfp-CMV infected cultures was visualized using a fluorescein isothiocyanate filter (green). The ICAM-1 (red) and GFP (green) images were superimposed, and the percent area of ICAM-1, GFP and overlap (yellow) was determined using Image-Pro Plus. To determine the percent area of high ICAM-1 expression, a baseline fluorescence was first selected based on the average fluorescence intensity of control cultures. The area of fluorescence which was

above the baseline fluorescence in infected or antibody treated ST cultures was then measured using Image-Pro Plus. The following formula was used to determine the percent area of high ICAM-1 expression: Percent area = (area of high ICAM-1 expression/total cell area) x 100.

5.2.6 TNFα and IL1β neutralization

To neutralize TNFα and IL1β released in trophoblast cultures, 20 μg/ml of polyclonal anti-human TNFα (Upstate Biotechnology; Lake Placid, NY), 20 μg/ml of polyclonal anti-human IL1β (R&D Systems; Minneapolis, MN) or both was added to the culture 2 hrs before virus challenge. After incubations, cells were washed with PBS three times, fixed with 4% paraformaldehyde, and ICAM-1 expression analyzed as described above.

5.2.7 Statistical analysis

See Chapter 3.2.7.

5.3 Results

5.3.1 HCMV induces ICAM-1 expression on the surface of ST cultures

In order to determine whether HCMV infection of ST cultures upregulated cell surface expression of ICAM-1, we carried out immunofluorescence analysis on paraformaldehyde-fixed cells treated with IFNγ, known to upregulate trophoblast ICAM-1 expression (Xiao *et al.* 1997), or challenged with HCMV for 24 hrs (Fig 5.1). As expected, there was much greater cell surface immunofluorescence on IFNγ-treated cells than on untreated cells (Fig 5.1A and B). HCMV infected cells had increased surface expression visually (Fig 5.1A), by digital analysis of fluorescence emissions (Fig 5.1B) from three independent experiments. In contrast, treatment with UV-inactivated virus and virus-free supernatant did not induce ICAM-1 expression suggesting that viral replication is required for upregulation.

5.3.2 HCMV-induced ICAM-1 expression is mediated by immediate early (IE) proteins

The rapid nature of HCMV-induced ICAM-1 expression indicates that an early viral event is responsible. Trophoblast cell surface interactions with virus coat proteins are the earliest possible event; however, it is unlikely that virus entry would induce ICAM-1 expression since UV-inactivated virus failed to do so. HCMV-induced ICAM-1 expression by HUVEC is mediated by viral IE genes (Burns *et al.* 1999). We therefore asked whether transcription of viral IE genes alone might induce trophoblast ICAM-1 levels.

HCMV IE promoter-driven IE expression plasmids carrying IE1-72, IE2-55, and IE2-86 (Yurochko *et al.* 1995) genes or the empty vector plasmid expressing only GFP were individually transfected into ST cultures, then cultured for 24hrs. A transfection frequency of 15%-25% was achieved as previously described (Chan *et al.* 2002). Following incubation, cell surface ICAM-1 expression was measured by immunofluorescence. All three IE proteins strongly induced ICAM-1 expression in the order of IE1-72>IE2-55>IE2-86, with IE1-72 stimulating expression almost as well as the positive control (IFNγ-stimulated cultures) (Fig 5.2).

The above photomicrographs (Fig 5.1A and 5.2A), combined with data from earlier experiments which show the infection frequency is <10%, indicate that ICAM-1 expression is rather widespread and thus suggest that cells other than those infected or transfected express higher levels of ICAM-1. To test this idea the distribution of ICAM-1 [cell surface (red)] and virus replication [GFP expression (green)] was determined by two color immunofluorescence (Fig 5.5A). GFP expression was driven from the early gene UL127 which is non-essential for replication in culture. The results indicate that ICAM-1 is expressed both in culture areas near and at sites of viral replication and therefore suggest some form of paracrine induction. However, they do not exclude concomitant direct induction.

5.3.3 TNFα mediates most of HCMV-induced ICAM-1 expression

We have previously shown that placental trophoblasts can release TNFα (Chan *et al.* 2002), that TNFα stimulates ICAM-1 expression in ST cultures (Xiao *et al.* 1997) and

that HCMV-IE protein expression stimulates TNFα release (Chan *et al.* 2002). Consequently, we asked whether HCMV-induced ICAM-1 was mediated by TNFα. Excess neutralizing antibody to TNFα was added to cultures at the time of HCMV infection or HCMV-IE transfection and cell surface ICAM-1 expression measured 24 hrs later by fluorescence emissions (Fig 5.3). The neutralizing TNFα antibody inhibited approximately 75% of HCMV and HCMV IE1-72 inducible ICAM-1 expression (total minus control) (Fig 5.3). Antibody to TNFα at 20 μg/ml is able to completely inhibit HCMV or IE-protein induced apoptosis mediated by this concentration of TNFα (Chan *et al.* 2002). We therefore conclude that 25% and 50% of HCMV and HCMV IE -induced ICAM-1 expression is independent of paracrine induction by TNFα.

HCMV-induced ICAM-1 expression in endothelial cells is partly mediated by secretion of IL1β (Woodroffe *et al.* 1993; Dengler *et al.* 2000). Consequently, we next examined whether IL1β accounts for the HCMV-induced ICAM-1 independent of TNFα. Excess neutralizing antibody to IL1β was added to the cultures during the time of challenge and ICAM-1 surface expression measured 24 hours later by fluorescence emissions (Fig 5.3). The addition of neutralizing IL1β antibody did not have any significant effect although a slight decrease was observed. Adding IL1β antibody in combination with TNFα antibody was marginally more effective than adding TNFα antibody alone; however, this again was not significant.

Although fluorescence emission gives an indication on the levels of ICAM-1 in the culture as a whole, it does not convey an impression on the distribution of ICAM-1. This can be had by analyzing the photomicrographs for the percent area of high ICAM-1 expression (higher than control cultures). As with fluorescence emission, the presence of neutralizing IL1β antibody did little to reduce the area of high ICAM-1 expression during a HCMV infection (Fig 5.4). However, unlike the fluorescence emission, IL1β antibody together with TNFα antibody was more effective than TNFα antibody alone at reducing the area of high ICAM-1 expression (Fig 5.4). Taken in combination, the data suggest that TNFα is the major mediator of paracrine HCMV-induced ICAM-1 expression while IL1β plays a minor role.

Neutralizing antibody was added in large excess, yet we were unable to completely block HCMV upregulation of ICAM-1 as determined by both fluorescence emission (Fig 5.3) and high expression area (Fig 5.4). The involvement of another cytokine is possible; however, images show large co-localization of ICAM-1 and viral replication suggesting a direct viral effect (Fig 5.5D).

5.3.4 HCMV-induced ICAM-1 expression is also a direct effect of virus

HCMV-induced ICAM-1 upregulation in human fibroblasts is direct and not mediated by inflammatory cytokine release (Ito *et al.* 1995; Craigen and Grundy 1996). In fibroblasts HCMV transactivators IE2-86 and pp71 synergistically induce ICAM-1 through the Sp1-binding site (Kronschnabl and Stamminger 2003). In order to determine whether only cells positive for HCMV replication express ICAM-1 after treatment with neutralizing antibodies, we carried out the infection experiments with gfp-CMV for 4 days, then asked whether there was any high ICAM-1 expression at a distance from GFP positive cells (Fig 5.5). Cultures not treated with neutralizing TNFα and IL1β antibodies exhibited areas of high ICAM-1 expression away from (red) and at (yellow) sites of viral replication. The presence of TNFα antibody reduced ICAM-1 expression not co-localized with GPF (Fig 5.5B) whereas antibody to IL1β had little effect (Fig 5.5C). The combination of both antibodies was slightly more effective at neutralizing paracrine ICAM-1 expression than TNFα antibody alone (Fig 5.5D).

We next quantitated the high ICAM-1 staining on cells infected [ICAM-1+/GFP+ (yellow)] and not infected [ICAM-1+/GFP- (red)] with HCMV. If the yellow [ICAM-1+/GFP+] area was unaffected by antibodies to TNFα and IL1β, the upregulation of ICAM-1 was due to direct effects of the virus. Alternatively, if the antibodies decrease this compartment, it would suggest that HCMV upregulates ICAM-1 expression in infected cells largely by autocrine release of TNFα or IL1β. We find that approximately 15% of the cell culture surface of ST cultures was positive for high ICAM-1 expression and HCMV and that neutralizing antibodies did not change this percentage (Fig 5.6A). Thus, ICAM-1 expression in infected cells was independent of the concentration of TNFα and IL1β in the supernatant. However, when the area of ICAM-1 not associated with infection (ICAM-1+/GFP-) was examined, it was reduced by the addition of

antibodies (Fig 5.6B). Antibodies to TNFα and IL1β both reduced ICAM-1 expression (the former greater than the latter) and together, they almost completely reversed the paracrine effect of HCMV. This showed that the ICAM-1 upregulation on uninfected cells was almost entirely due to the release of TNFα and IL1β by the infection.

5.4 Discussion

We hypothesized that ICAM-1 would be strongly expressed by the ST after HCMV infection. In this study villous CT (by criterion of absent MHC class I, class II and CD9) were differentiated to an ST culture with EGF (Guilbert *et al.* 2002). Until induction with HCMV (and IFNγ), ST cultures expressed little ICAM-1. However, after induction with the virus, they expressed high levels on their apical surfaces within 24 hrs of exposure. Thus, HCMV infection of the villous ST can initiate an inflammatory cascade which could lead to enhanced monocyte binding to the ST surface and thereby inducing ST damage (Xiao *et al.* 1997; Garcia-Lloret *et al.* 2000). This is the first report showing that HCMV infection induces ICAM-1 expression in primary trophoblast cultures.

HCMV exposure to vascular endothelial cells upregulates ICAM-1 (Scholz *et al.* 1996; Shahgasempour *et al.* 1997; Burns *et al.* 1999; Dengler *et al.* 2000; Kronschnabl and Stamminger 2003). However, whether the upregulation is due to virus alone or mediated by paracrine release of IL1β is controversial (Scholz *et al.* 1996; Burns *et al.* 1999; Dengler *et al.* 2000). Since villous trophoblasts are also vascular cells and upregulation of ICAM-1 on these cells can be induced by TNFα, IL1β, and IFNγ (Xiao *et al.* 1997), we asked whether the upregulation of ICAM-1 by HCMV was paracrine (mediated by cytokines) or direct. We found that < 15% of the cells were infected but that > 30% of the surface area expressed elevated levels of surface ICAM-1. Adding antibody to TNFα reduced the surface area expressing ICAM-1 by greater than half but left approximately 15%. When examining cells not infected with HCMV, we determined that IL1β antibody could reproducibly reduce the expression of ICAM-1 alone, and in combination with antibody to TNFα, almost completely reversed paracrine induction. This indicated that HCMV upregulation of ICAM-1 on cells not infected with HCMV took place by paracrine release of TNFα and IL1β. What this means *in vivo* is

complicated by the ST being a single, continuous cell. The infected portion of the ST could secrete TNFα and IL1β which in turn could act at a distance to induce ICAM-1. However, this remains to be proven and it is unclear how much this mechanism contributes to ICAM-1 expression *in vivo*.

In contrast, our results showing that high ICAM-1 expression on infected cells was significant and unaffected by antibody to TNFα and IL1β provides an unambiguous way for HCMV-1 to upregulate ICAM-1 on intact ST. Presumably, the autocrine contributions of the cytokines are subsumed by the direct effect of HCMV infection on ICAM-1 expression. A direct effect of HCMV on ICAM-1 expression is in accord with earlier observations in fibroblasts of HCMV IE2-86 and pp71 inducing ICAM-1 through the SP1 site (Kronschnabl and Stamminger 2003). However, this is the first report that HCMV both stimulates expression in a paracrine manner via cytokine release and directly in infected cells.

The rapid nature of HCMV-induced ICAM-1 expression suggests that an early event is responsible. Virus entry is the earliest event in the virus life cycle and has been shown to regulate transcription of a number of cellular genes in the absence of viral replication (Yurochko *et al.* 1995; Yurochko *et al.* 1997; Zhu *et al.* 1998; Boyle *et al.* 1999; Browne *et al.* 2001; Simmen *et al.* 2001) via cellular signaling receptors such as TLR2 (Compton *et al.* 2003). Binding of the virus upregulates a number of transcription factors including Sp1 (Yurochko *et al.* 1997), which is known to regulate the promoter of ICAM-1 (Kronschnabl and Stamminger 2003). However, we have found that UV-inactivated virus does not alone stimulate significant surface ICAM-1 suggesting that viral gene transcription and translation are required. We have also shown that transfection with the viral immediate early genes IE-72, IE-55 and IE-86 upregulates ICAM-1 expression. This indicates that virus gene transcription to the immediate early stage is necessary and sufficient for ICAM-1 induction.

HCMV infection of placental trophoblast is associated with villous inflammation (villitis) (Garcia *et al.* 1989; Sinzger *et al.* 1993; Bernstein and Divon 1997; Benirschke and Kaufmann 2000) which is characterized by the focal loss of villous trophoblast (Benirschke and Kaufmann 2000). We have previously suggested a mechanism whereby HCMV infection stimulates TNFα release and subsequently the rapid induction of

apoptosis in uninfected neighboring cells (Chan *et al.* 2002). This loss of trophoblasts would contribute to HCMV-related placental villits. However, in this communication, we suggest an alternative, and perhaps complementary, mechanism. There is an accumulation of mononuclear phagocytes within placental lesions associated with HCMV (Greco *et al.* 1992; Kohut *et al.* 1997) and we have shown that binding of monocytes to cultured ST via ICAM-1 leads to TNFα-mediated ST damage (Garcia-Lloret *et al.* 2000). Infection would induce ICAM-1 expression on the ST and this would bind monocytes to the ST. Thus, induction of ICAM-1 on the villous ST by HCMV infection could be an early step in trophoblast loss characteristic of villitis.

Our current studies also have major implications for possible mechanisms of *in utero* transmission of HCMV from mother to fetus across the placenta. Infected villous trophoblasts release less than 5% of progeny virus (Hemmings *et al.* 1998). Furthermore, progeny virus from productively infected polarized ST membrane cultures is released from the apical surface (toward the maternal circulation) (Hemmings *et al.* 2001). Taken together, these observations do not support an infection and release model of vertical transmission. An alternative model is one where vertical transmission occurs when the ST barrier has been damaged and thus compromised. Release of TNFα due to HCMV infection can directly deplete underlying CT that have not yet been infected (Chan *et al.* 2002). In this study we show that infection of ST cultures leads to ICAM-1 expression, which in turn could lead to monocyte binding (Xiao *et al.* 1997) and localized loss of the ST (Garcia-Lloret *et al.* 2000). The combination of ST loss by monocyte binding and CT loss by release of TNFα can lead to local rupture of the villous trophoblast by HCMV. Such a model suggests that vertical transmission is secondary to villous trophoblast damage and is consistent with correlations between *in utero* transmission and placental villitis (Benirschke *et al.* 1974).

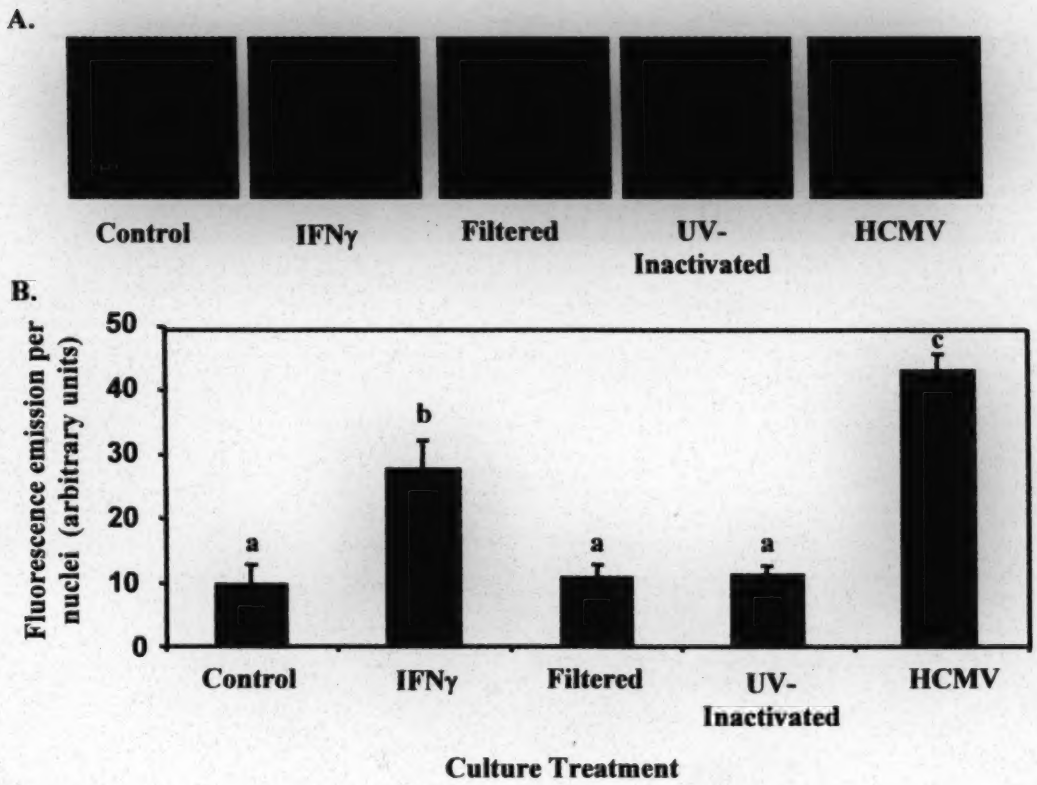

Figure 5.1: Immunofluorescence of cell surface ICAM-1 from ST cultures after treatment with IFNγ and HCMV.

Panel A: Photomicrographs of a single experiment in which ST cultures were either untreated (control) or treated for 24 hrs with IFN (100 U/ml) or challenged at an MOI of 10 with a HCMV preparation that had been filtered through a 0.1 μm filter to remove virus particles (filtered), UV-inactivated or not treated (HCMV). Panel B: Digital analysis of fluorescence emissions shown in panel A. Depicted are the average $\pm$ SD of emissions (expressed as arbitrary units from three independent experiments carried out on two different placental preparations). Emission units in the different cultures have been normalized to the number of nuclei (determined by DAPI staining) per image. Experimental groups within each panel labeled with different letters (a, b or c) are significantly different ($P<0.05$).

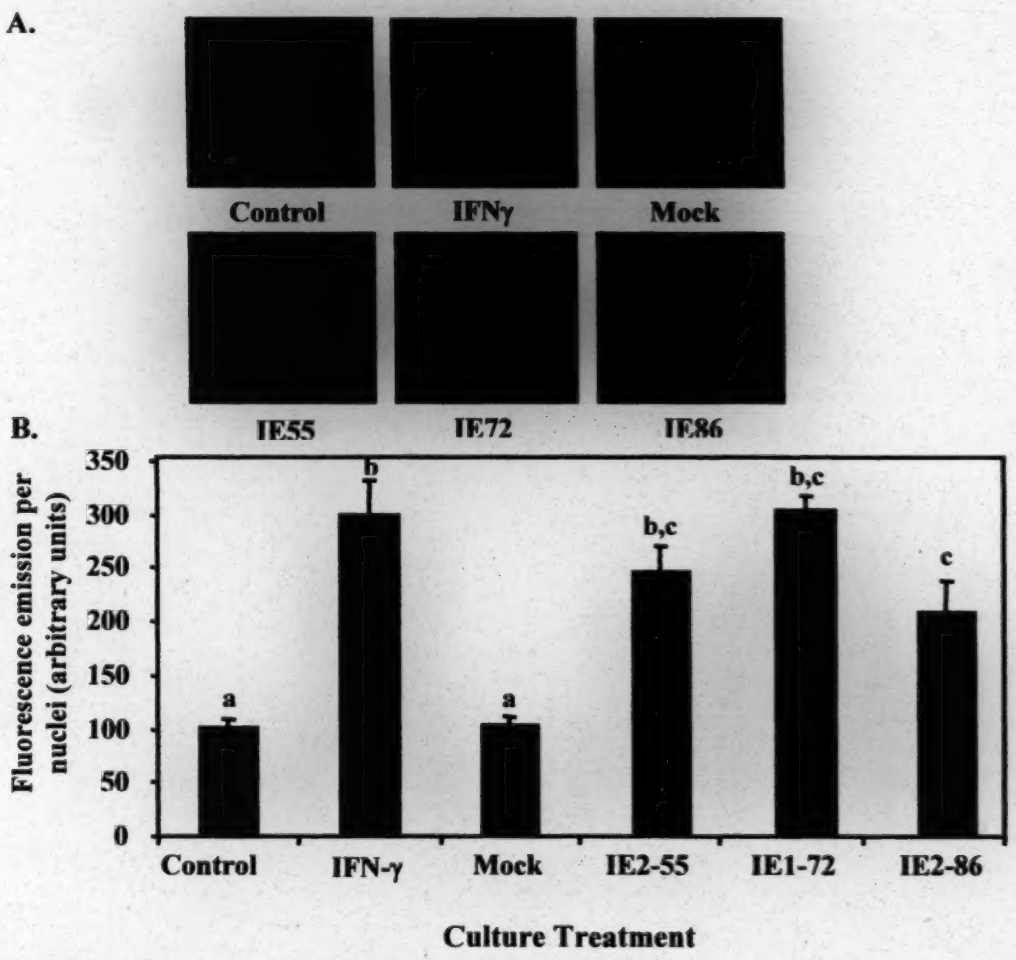

Figure 5.2: Immunofluorescence of cell surface ICAM-1 from ST cultures after no treatment (control), treatment with IFNγ or transfection with an empty expression plasmid (mock), or with expression plasmids containing HCMV-IE2-55, IE1-72 or IE2-86 genes.

Panel A: Photomicrographs of trophoblast cultures after the indicated treatments. Panel B: Digital analysis of fluorescence emission shown in panel A. Depicted are the average $\pm$ SD of emissions (expressed as arbitrary units) from three independent experiments carried out on two different placental preparations. Emission units in the different cultures have been normalized to the number of nuclei (determined by DAPI staining) per image. Experimental groups within each panel labeled with different letters (a, b or c) are significantly different ($P<0.05$).

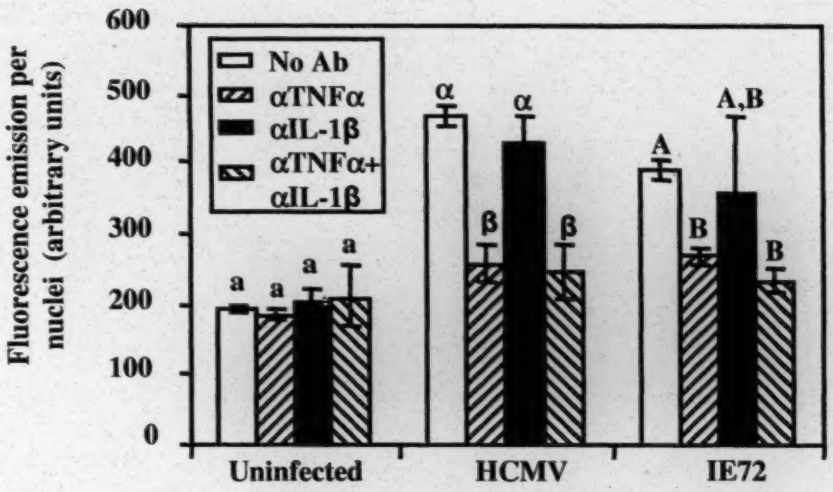

Figure 5.3: The effect of neutralizing anti-TNFα antibody and anti-IL1β antibody on HCMV and HCMV-IE protein induced ICAM-1 expression in trophoblasts as measured by fluorescence emissions.

ST cultures were not treated (control), infected with HCMV (MOI 10) or transfected with HCMV IE1-72, cultured with or without 20 μg/ml neutralizing antibody to TNFα or IL1β for 24 hrs then assessed for cell surface ICAM-1 expression by immunofluorescence. Depicted are the average ± SD of emissions (expressed as arbitrary units) from three independent experiments carried out on two different placental preparations. Emission units in the different cultures have been normalized to the number of nuclei (determined by DAPI staining) per image. Experimental groups within each panel labeled with different letters (a, b or c for uninfected, α or β for HCMV infected, A or B for IE72 transfected) are significantly different ($P<0.05$).

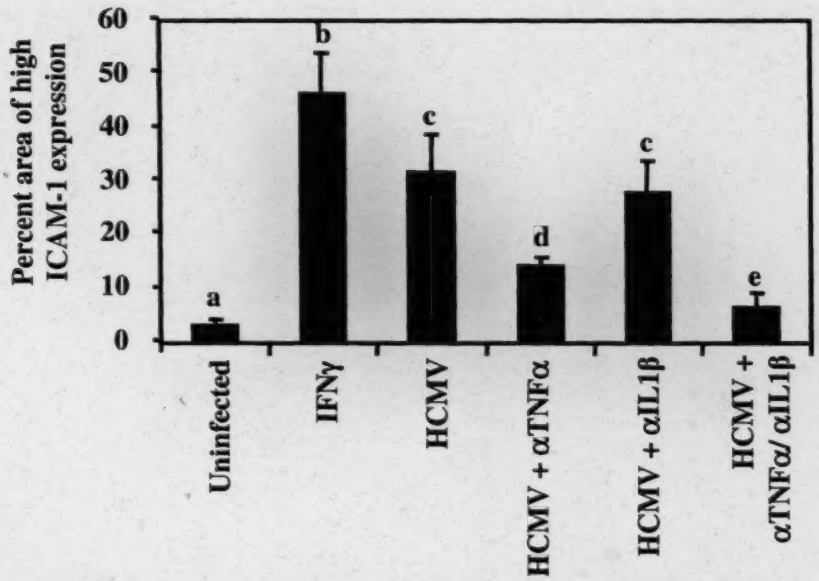

Figure 5.4: The effect of neutralizing anti-TNFα antibody and anti-IL1β antibody on HCMV-induced ICAM-1 expression on trophoblasts as measured by percent area of high ICAM-1 expression in the culture.

ST cultures were not treated (control), infected with HCMV (MOI 10), or cultured with or without 20 μg/ml neutralizing antibody to TNFα or IL1β for 24 hrs then assessed for the percent surface area of high ICAM-1 expression in the cell culture. Depicted are the average ± SD percent area of high ICAM-1 expression from three independent experiments carried out on two different placental preparations. Experimental groups within each panel labeled with different letters (a, b, c, d or e) are significantly different ($P<0.05$).

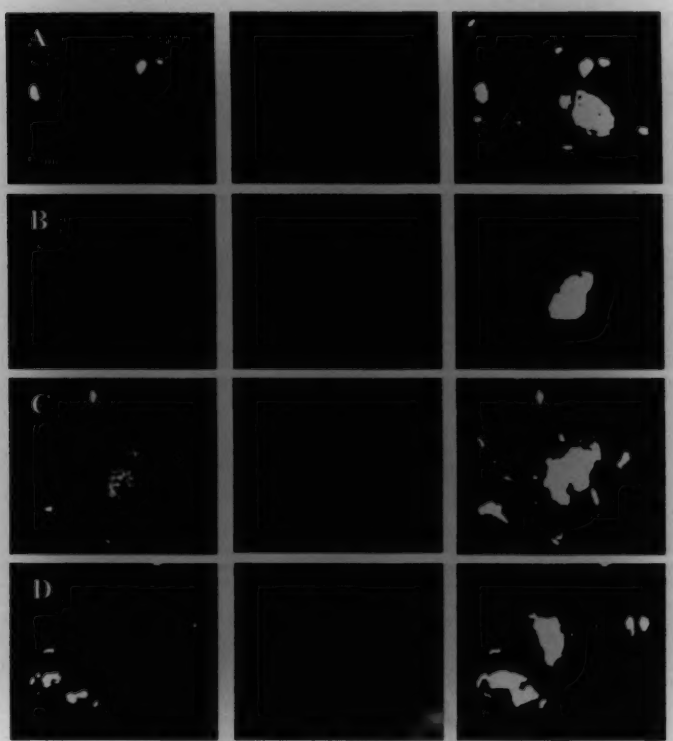

Figure 5.5: Immunofluorescence of cell surface ICAM-1 (red) and GFP expression (green) from ST cultures infected with gfp-HCMV (MOI 10).

Panel A: Photomicrographs of trophoblast cultures infected with HCMV. Panel B: Photomicrographs of trophoblast cultures infected with HCMV in the presence of 20 μg/ml of anti-IL1β antibody. Panel C: Photomicrographs of trophoblast cultures infected with HCMV in the presence of 20 μg/ml of anti-TNFα antibody. Panel D: Photomicrographs of trophoblast cultures infected with HCMV in the presence of 20 μg/ml of anti-TNFα antibody and anti-IL1β. Overlay images depicting ICAM-1 and GFP co-localization (yellow) were done in Adobe Photoshop 6.0.

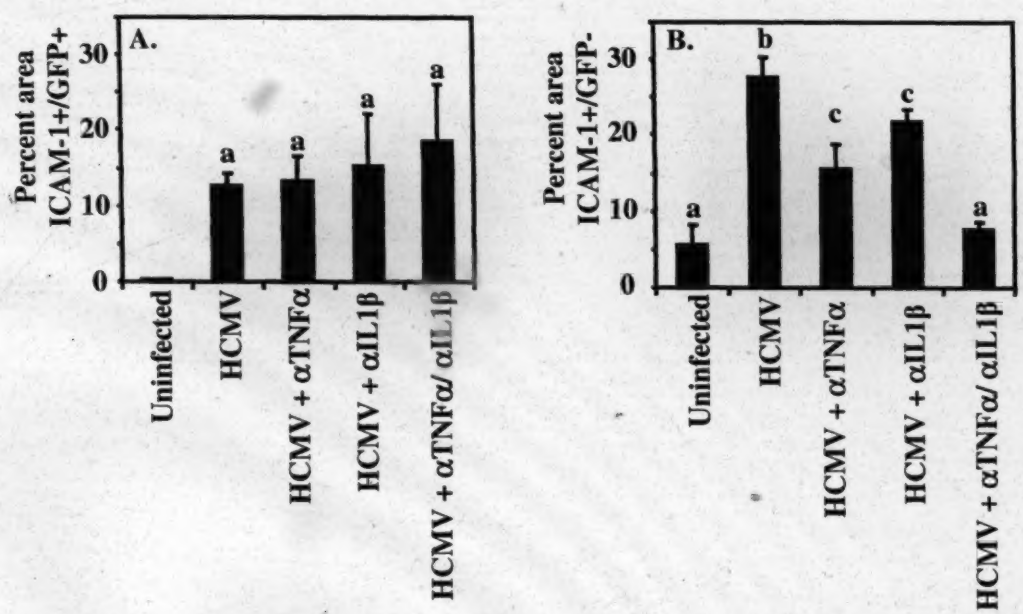

Figure 5.6: The effect of neutralizing anti-TNFα antibody and anti-IL1β antibody on HCMV-induced ICAM-1 expression co-localized (ICAM-1+/GFP+) or not co-localized (ICAM-1+/GFP-) to sites of viral replication (GFP expression).

ST cultures were either untreated (control) or challenged with gfp-HCMV (MOI 10) for 4 days in the presence of 20 μg/ml of anti-TNFα antibody, anti-IL1β antibody or both. Depicted are the average ± SD of the percent area of ICAM-1+/GFP+ (A) and ICAM-1+/GFP- (B) from three independent experiments carried out on two different placental preparations. Experimental groups within each panel labeled with different letters (a, b or c) are significantly different ($P<0.05$).

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CHAPTER 6: ENHANCED MONOCYTE BINDING TO HUMAN CYTOMEGALOVIRUS INFECTED SYNCYTIOTROPHOBLAST RESULTS IN INCREASED APOPTOSIS VIA THE RELEASE OF TUMOR NECROSIS FACTOR α

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6.1 Introduction

Morphological studies indicate that congenital HCMV infection is associated with a number of changes including focal inflammation of the villous placenta and loss of the trophoblast (Benirschke *et al.* 1974; Benirschke and Kaufmann 2000). There is an accumulation of mononuclear phagocytes within these inflammatory lesions (Garcia *et al.* 1989; Greco *et al.* 1992). In culture the inflammatory cytokines IL1, TNFα and IFNγ can stimulate monocyte adhesion to an ST surface by upregulating ICAM-1 (Xiao *et al.* 1997). Infection of these cultures by HCMV induces surface ICAM-1 expression indirectly on uninfected cells by release of IL1β and TNFα from infected cells and directly on infected cells (Chan *et al.* 2004). IFNγ-activated ST cultures bind monocytes which induce trophoblast damage in a TNFα-dependent manner (Garcia-Lloret *et al.* 2000). However, it is unknown whether HCMV infected ST upregulates monocyte binding and given the resistance of HCMV-infected cells to TNFα-induced apoptosis, whether this binding would lead to increased trophoblast apoptosis.

We therefore hypothesize that HCMV infection of ST leads to enhanced monocyte binding which leads to the induction of ST apoptosis. Using highly purified (>99.99%) CT differentiated into ST cultures with EGF, we found that HCMV infection enhanced monocyte adhesion to HCMV-infected cultures, that binding was by ICAM-1 (on trophoblasts) and LFA-1 (on monocytes) and that the increased monocyte binding led to elevated levels of apoptosis mediated by TNFα.

6.2 Methods and Materials

6.2.1 Cells

See Chapter 3.2.1.

6.2.2 Virus Preparation, Culture Challenge, and Assessment of Infection

See Chapter 5.2.2.

6.2.3 Preparation of Monocytes

Fresh peripheral blood was centrifuged over Histopaque-1077 (Sigma) and the interface band saved. The cells were then washed twice with IMDM, resuspended in 10% FBS-IMDM, and incubated at 37°C in 100-mm^2 tissue culture dishes (Corning 25020, Fisher Scientific, Edmonton, Canada) for 4 hrs. Non-adherent cells in medium were decanted, the dishes were washed twice with IMDM to remove residual non-adherent cells, and fresh 10% FBS-IMDM was added. After overnight culture, the adherent cells were collected by vigorous pipetting. Greater than 90% of the harvested cells were monocytes as determined by CD11c immunohistochemistry (see below). All monocytes were activated by incubation with 0.5 μg/ml LPS (Sigma, catalog number L2880) for 2 hrs before harvesting for co-culture with trophoblasts.

6.2.4 Monocyte Adhesion Assay

Trophoblasts were cultured on microtiter plates as described above. Following a 5 day incubation with EGF, ST cultures were treated with control medium, IFNγ (100 U/ml), or infected with AD169 or Kp7 as described above and the culture continued for another 24 hrs, at which time cultures were twice washed with warm 2% FBS-IMDM. ST cultures infected with gfp-CMV were washed five times on day 1 after infection with warm 2% FBS-IMDM, 10% FBS-IMDM containing EGF was added (and changed at day 3) and continued to day 4 (GFP expression can be seen at this time), then media changed back to 2% FBS-IMDM. Activated monocytes (2 x 10^6/well) were added to all trophoblast cultures. After varying periods of incubation at 37°C, unbound monocytes were removed by washing gently with warm PBS three times. Tightly bound monocytes were cross-linked for 20 min at room temperature to trophoblasts with ethylene glycol bis

succinic acid N-hydrosuccinimide ester (EGS; Sigma) made fresh as 0.2 M stock dissolved in DMSO, then diluted 1/25 in fixative buffer (100mM KCl, 5mM MgCl2, 2.5% glycerol, 20 mM HEPES-KOH, pH 7.5). Trophoblasts were then washed three times with PBS, fixed with methanol for 10 min at -20°C and stored in PBS until immunofluorescence staining for CD45 (to detect monocytes) and/or TUNEL (to detect apoptosis) was carried out (see below for details).

For adhesion blocking experiments, anti-ICAM-1 antibody (ICOS Corporation, Bothell, Washington, 10 μg/ml)) or anti-LFA-1 (Immunotech; Westbrook, ME, 10 μg/ml) antibody was added to trophoblasts or monocytes, respectively, and incubated for 1 hr at 37°C. The trophoblasts were then washed with IMDM and monocytes were added as described above. Neutralizing antibodies were also present during the two hour binding period. All blocking experiments were also carried out with isotype-matched IgG.

6.2.5 Immunofluorescence Staining

Two-color fluorescence analysis was carried out in parallel cultures to determine total nuclei number and the fraction of nuclei expressing HCMV IE proteins. After methanol fixation and PBS washing, non-specific binding sites were blocked with 3% skim milk/0.5% Tween 20/PBS at 20°C for 30 min. Primary antibodies detecting HCMV IE (detecting p72, p55 and p86; Specialty Diagnostics, Dupont) or its isotype control, IgG1 (Dako Corporation) were added and incubated at 20°C for 1 hr. The primary antibody was then removed and the cells washed five times with PBS. Next, 50 μl of Alexa Fluor 546 goat anti-mouse IgG conjugate (Molecular Probes) in 3% skim milk/0.5% Tween 20/PBS at 1 μg/ml was added to each well for 60 min at 20°C. The cells were washed with PBS five times and nuclei visualized with 100 μl of 1.4 μg/ml DAPI (Molecular Probes, 10 min, 20°C). The frequency of infection was determined by the number of IE-positive nuclei (red fluorescence) relative to the total number of nuclei (blue).

For analysis of apoptosis and monocyte binding to AD169-infected cells, three color fluorescence analysis was carried out. After methanol fixation and PBS washing, the fraction of nuclei with nicked DNA was determined by TUNEL (Gavrieli *et al.* 1992)) as previously described in (Yui *et al.* 1994; Chan *et al.* 2002). Briefly, after the

reaction was terminated by adding 2X SCC, the cells were washed three times with double-distilled water, nonspecific binding sites blocked with non-immune goat serum (Zymed Laboratories; Markham, CA) for 30 min and primary antibody to CD45 (Biosource International; Camarillo, CA) or isotype control, IgG1 (Dako Corporation; Carpinteria, CA) added and incubated at 20°C for 1 hr. The primary antibody was then removed, the cells washed five times with PBS and 50 µl per well of streptavidin Alexa Fluor 488 conjugate (Molecular Probes; Eugene, OR) and Alexa Fluor 546 goat anti-mouse IgG conjugate (Molecular Probes) diluted in PBS to 1 µg/ml added and incubated for 1 hr at 20°C. Cells were then washed with PBS five times and 100 µl of 1.4 µg/ml DAPI (Molecular Probes) added to each well for 5 min to visualize all nuclei. The total number of nuclei (DAPI, blue), CD45-positive (Alexa Fluor 546, red), and TUNEL-positive (Alexa Fluor 488, green) were determined per well by digital analysis as described below.

For analysis of apoptosis and monocyte binding to gfp-HCMV infected cells, three color fluorescent analysis was carried out as described above with the following modification: streptavidin Alexa Fluor 350 conjugate (Molecular Probes) was used to detect TUNEL-positive cells and Alexa Fluor 546 goat anti-mouse IgG conjugate to detect CD45-positive cells. Fluorescent microscopy was used to visualize TUNEL-positive nuclei (Alexa Fluor 350, blue) CD45-positive (Alexa Fluor 546, red), and infected cells (GFP-positive, green) as described below.

6.2.6 Digital photography and analysis

Fluorescence images were obtained with an Olympus IX2-UCB motorized inverted research microscope equipped with a Lambda DG-4 high speed filter changer and a Cascade 16 bit digital monochrome camera (Olympus, Melville, NY). Digital images of each well were obtained with DAPI (blue) and rhodamine (red) filters. We used Slidebook 3.0 (Carsen, Markham, ON, Canada) as capture software and Image Pro-Plus (Media Cybernetics; Del Mar, CA) for analysis. The blue, red and green images were superimposed using an imaging program, Image-Pro Plus (Media Cybernetics; Del Mar, CA). For monocyte adhesion experiments, three wells were examined per treatment in each independent experiment and the number of CD45-positive cells determined after

examination of a minimum of 1200 nuclei per well. Similarly, the percentage of TUNEL-positive nuclei was determined in triplicate wells after examination of a minimum of 1200 nuclei per well.

6.2.7 RNA extraction and reverse-transcriptase polymerase chain reaction (RT-PCR)

For analysis of CD45 gene expression, total RNA from ST, treated with medium, IFNγ or infected with HCMV and co-cultured with monocytes were extracted with Trizol (GIBCO) as described by the manufacturer. Total RNA extracted was resuspended in sterilized distilled water. RNA concentration was determined by measuring absorbance at 260 nm.

Reverse transcription was performed on 2 μg of total RNA using the TaqMan reverse transcription (RT) kit (Invitrogen), containing 1 X RT buffer (Invitrogen RT buffer), 5.5 mM $MgCl_2$, 500 μM deoxyNTP, 2.5 μM random primers, 0.4 U/μl RNase inhibitor, and 1.25 U/μl reverse transcriptase in a total volume of 40 μl. Control mixtures were prepared for each RNA sample containing no reverse transcriptase and a no-template control was also included. cDNAs were amplified using 2 U of Taq polymerase (Invitrogen) in a 30 μl reaction volume containing 10 μM of CD45 specific primers, 10 μM of each deoxynucleoside triphosphate, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, and 1.5 mM $MgCl_2$. Primer pairs specific for CD45 (sense, 5'-GGAAGTGCTGCAATGTGTCATT-3'; antisense, 5'-CTTGACATGCATACTATTATCTGA- TGTCA-3') were purchased from DNA Core Services Laboratory (University of Alberta, Edmonton, Canada). The cycling parameters were as follows: initial denaturation at 95°C for 10 min then 35 cycles at 95°C for 15 s, and 60°C for 1 min. Amplification of GAPDH was used as a control. The PCR products were electrophoresed on a 1.5% agarose gel and detected with ethidum bromide.

6.2.8 Analysis of DNA fragmentation

ST cultures were lysed *in situ* with 0.5 ml of lyis buffer (50 mM Tris pH 7.5, 20 mM EDTA, 1% NP40) for 15 min on ice. The lysates were transferred to microcentrifuge tubes and treated with proteinase K (100 μg/ml) for 12 hrs at 37°C. Digests were spun for 8 min at 5000 rpm to remove cellular debris and the supernatants

treated with DNAse free RNAse for 2 hrs at 37°C. Isolated DNA was then precipitated with 3 M sodium acetate and dissolved in sterilized distilled water. Electrophoresis was performed on samples in 1.5% gels in TBS (0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA buffer) for 2 hrs at 100 V. Agarose gels were stained with 10 ng/ml ethidium bromide for 4 hrs.

6.2.9 TNFα Neutralization

To neutralize TNFα released in trophoblast cultures, 10 μg/ml of polyclonal anti-human TNFα (Upstate Biotechnology; Lake Placid, NY) was added to the culture 2 hrs before virus challenge. Activated monocytes were then added to infected cultures as described above in the presence of anti-TNFα. Following 24 hr incubation, cells were washed with PBS three times, fixed and analyzed as described above.

6.2.10 Statistical Analysis

See Chapter 3.2.7.

6.3 Results

6.3.1 Monocytes have increased adhesion to HCMV-infected ST cultures

We have previously shown ICAM-1 to be upregulated on HCMV-infected ST cultures (Chan *et al.* 2004). In order to determine whether HCMV-stimulated upregulation of ICAM-1 leads to increased monocyte adhesion, we examined the number of bound monocytes at different time points on ST cultures treated with control media, IFNγ (known to upregulate trophoblast ICAM-1 expression and monocyte adhesion (Xiao *et al.* 1997)), or infected with HCMV (Fig 6.1A). Trophoblasts do not express the common leukocyte antigen (CD45); thus, we detected bound monocytes by immunofluorescence. Monocyte adhesion is slow but after 2 hrs, the number bound was significantly higher in IFNγ-treated and HCMV-infected cultures than in control cultures. Furthermore, more monocytes bound to HCMV-infected cells than to IFNγ-treated cultures. We confirmed HCMV- and IFNγ-induced monocyte adhesion by determining the levels of CD45 mRNA in these cultures. We see a significant increase in the expression of CD45 mRNA in co-cultures where ST were treated with IFNγ or infected

with HCMV (Fig 6.1C). Challenging ST cultures with a clinical variant of HCMV (Kp7) for 24 hrs also resulted in increased monocyte adhesion to these cultures (Fig 6.1B) albeit to a lesser extent than AD169 (compared to Fig 6.1A).

6.3.2 Monocyte binding is mediated by ST ICAM-1 interaction with monocyte LFA-1

To determine whether trophoblast ICAM-1 mediated HCMV-induced monocytes binding, adhesion was blocked with neutralizing ICAM-1 antibody. This antibody reduced monocyte binding to HCMV-infected ST cultures to the level of the uninfected cultures (Fig 6.2) suggesting that increased monocyte binding was due to elevated levels of ICAM-1 on the cell surface (Chan *et al.* 2004). Since LFA-1 is a ligand of ICAM-1 (Marlin and Springer 1987) and has been shown to mediate monocyte binding to trophoblasts (Garcia-Lloret *et al.* 2000), we next asked what effect monocytes pre-treatment with LFA-1 antibody would have on HCMV-induced binding. LFA-1 antibody reduced monocyte adhesion to infected cultures as much as treatment of trophoblasts with anti-ICAM-1 antibody (Fig 6.2) suggesting that LFA-1 was the ICAM-1 ligand on monocytes.

6.3.3 Monocytes stimulate apoptosis in ST cultures

Monocytes adhering to ST cells induce focal apoptosis and loss of cells (Garcia-Lloret *et al.* 2000) but HCMV-infected cells are resistant to cytokine-stimulated apoptosis (Chan *et al.* 2004). Therefore, we next asked if increased HCMV-induced monocyte adherence stimulated trophoblast apoptosis. In uninfected cultures, the presence of monocytes did not significantly induce apoptosis (Fig 6.3). HCMV infection alone has been shown to induce trophoblast apoptosis (Chan *et al.* 2002). In agreement, our results show HCMV to increase apoptosis rates from ~6% to ~13%. However, co-incubation of monocytes with infected ST cultures further increased apoptosis to ~19%. There was also a significant increase in DNA fragmentation found in HCMV-infected ST co-cultured with monocytes (Fig 6.4B, lane 7). Regions that had clusters of bound monocytes exhibited elevated levels of apoptosis. Figure 6.5D shows a typical cluster of apoptotic (TUNEL-positive) nuclei in the vicinity of a number of trophoblast-bound monocytes (CD45-positive).

To determine whether monocyte adhesion caused trophoblast apoptosis, the interaction was blocked with antibodies to ICAM-1 and LFA-1. Preincubation of ICAM-1 antibody with HCMV-infected ST cultures and LFA-1 antibody with activated monocytes was able to completely abrogate monocyte-induced apoptosis (levels of apoptosis in ICAM-1/LFA-1-blocked cultures were similar to those of the HCMV-infected cultures, Fig 6.4A).

We have previously shown that HCMV induced ICAM-1 expression both on infected and, in a paracrine fashion, on uninfected trophoblasts (Chan *et al.* 2004). In similar fashion, we here find that monocytes bound to both infected and uninfected trophoblasts (Fig 6.6). Further, regions of the ST infected with HCMV did not undergo apoptosis even when bound to monocytes (Fig 6.6). Thus, these results suggest that monocyte binding leads to apoptosis but only to uninfected cells. Upon examination of ~11000 cells per treatment, none of the infected cells were undergoing apoptosis, but 21% of uninfected cells were undergoing apoptosis ($p<0.0001$).

6.3.4 Monocyte-induced apoptosis is mediated by TNFα release

HCMV-mediated trophoblast apoptosis is dependent on the secretion of TNFα (Chan *et al.* 2004). In order to confirm that the apoptosis seen in monocyte adhesion to HCMV-infected cultures was TNFα-dependent, neutralizing antibody was added to the co-cultures. The presence of TNFα antibody reduced apoptotic rates to similar levels found in cultures infected only with HCMV (Fig 6.4A, compare lanes 4 and 6). This reduction in apoptosis was not due to TNFα antibody inhibition of HCMV infection or HCMV-induced ICAM-1 expression (Chan *et al.* 2004) since the cells were infected with HCMV for 24 hrs in the absence TNFα antibody and surface ICAM-1 expression is stable on trophoblasts for >48 hrs (Xiao *et al.* 1997). Further, anti-TNFα antibody had no effect on monocyte adhesion (Fig 6.2). In order to determine whether we could completely block HCMV-induced, monocyte-mediated apoptosis (to the level of uninfected cultures with no monocytes), we added EGF during the first 24 hrs of infection. This blocked HCMV-induced apoptosis but not ICAM-1 induction [EGF blocks TNFα and HCMV-induced apoptosis (Garcia-Lloret *et al.* 1996; Chan *et al.* 2002) but does not affect TNFα and IL-1-induced ICAM-1 expression (Xiao *et al.* 1997)].

After infection and monocyte addition, the apoptosis frequency (Fig 6.4A, compare lanes 3 and 5) and the amount of DNA fragmentation (Fig 6.4B) was not significantly different from control cultures. Similarly, we found that the addition of EGF, a known inhibitor of TNFα induced trophoblast apoptosis (Garcia-Lloret *et al.* 1996), instead of anti-TNFα during monocyte co-culture also reduced apoptosis to control culture levels (Fig 6.4A, compare lanes 3 and 9). Digital analysis indicated that the presence of anti-TNFα blocked the strong induction of apoptosis to the trophoblast underlying monocyte clustering (Fig 6.5E and 6.5F). These observations indicate that monocytes were inducing apoptosis in a mechanism dependent on TNFα release.

6.4 Discussion

Monocyte adherence to ST cultures depends on ICAM-1 (on the trophoblast) to LFA-1 (on the monocyte) interactions (Xiao *et al.* 1997). Since HCMV infection of these cultures increased ICAM-1 expression indirectly in uninfected cells by paracrine release of the inflammatory cytokines TNFα and IL1β from infected cells and directly in a cytokine-independent manner on infected cells (Chan *et al.* 2004), we predicted that HCMV infection would increase monocyte binding to ST cultures. In this study, we tested that prediction by infecting ST cultures with HCMV and then adding LPS activated monocytes. We found a higher binding capacity for monocytes and confirmed with neutralizing antibodies that the increase in adhesion was mediated by ICAM-1 on the ST culture and LFA-1 on the monocytes. These results support our prediction that HCMV infection upregulates monocyte binding to villous trophoblasts in an ICAM-1-dependent fashion.

We have previously shown that monocyte binding to uninfected trophoblasts increased trophoblast apoptosis (Garcia-Lloret *et al.* 2000). In that study the cultures were first treated with IFNγ which increased ICAM-1 expression and thus increased monocyte binding and subsequent trophoblast damage. In the present study the uninfected cultures used to determine apoptosis were not treated with IFNγ, thus didn't express high levels of ICAM-1 and therefore did not bind monocytes which increased apoptosis.

Increased monocyte binding increases the frequency of apoptosis in ST cultures when IFNγ is present (Garcia-Lloret *et al.* 2000), suggesting that the HCMV-caused increase in monocyte binding might increase apoptosis. Our results support this hypothesis. Although infected ST cultures alone show elevated levels of apoptosis, those co-cultured with adhering monocytes show an additional increase. Furthermore, blocking monocyte adhesion using anti-ICAM-1 or LFA-1 antibody resulted in a reduction of apoptosis to similar levels as infected cultures but not to uninfected control cultures. However, the additions of EGF during HCMV infection and anti-TNFα during monocyte adhesion were able to reduce apoptosis of infected ST cells co-cultured with monocytes to levels similar to uninfected control cultures. EGF present during the 24 hr infection period blocked HCMV-induced apoptosis as previously observed (Chan *et al.* 2002). TNFα antibody could not be used at this stage to block HCMV-induced apoptosis as the action of TNFα partly accounts for HCMV-induced ICAM-1 expression (Chan *et al.* 2004). The presence of TNFα antibody during monocyte co-culture does not affect HCMV-stimulated ICAM-1 surface expression since ICAM-1 expression is stable on trophoblasts for >48 hrs (Xiao *et al.* 1997) nor does it interfere with monocyte adhesion to the ST surface (Fig 5.2). These observations show that HCMV not only increases trophoblast apoptosis alone but that it also upregulates ICAM-1, which can bind monocytes that in turn further increase the frequency of apoptosis. *In vivo* HCMV infection is often associated with placental inflammation, which is accompanied by the focal loss of the trophoblast. The present results are in accord with the hypothesis that the pathology of ST infection by HCMV can be locally amplified by infection-induced upregulation of ICAM-1 and transient binding of maternal monocytes.

During a HCMV infection, monocytes are the primary cell type infected in blood (Taylor-Wiedeman *et al.* 1991; Sinclair and Sissons 1996; Sinzger and Jahn 1996) and are the predominant infiltrating cell found in infected brains (Booss *et al.* 1989). HCMV-infection of monocytes is not productive but non-productive infection induces cellular changes that promote transendothelial cell migration, differentiation and permissive viral replication (Smith *et al.* 2004). There are twice as many monocytes per unit volume of blood in the intervillous space as in peripheral blood (Moore *et al.* 2003) suggesting a low affinity interaction between monocytes and the villous ST. This interaction could

allow infected monocytes to adhere, mature and occasionally transfer an infection to a susceptible region of the ST. Alternatively, if the ST were already infected by an intervillous route (Fisher *et al.* 2000), the interaction would be enhanced by upregulation of ICAM-1. Regions of the ST infected by either villous or extravillous routes would include those near the capillary endothelium where oxygen levels are low (Benirschke and Kaufmann 2000) and transcription is still active (Huppertz *et al.* 2003). Interestingly, the infection could be abortive since IE gene expression alone will induce ICAM-1 on ST-like cells (Chan *et al.* 2004). However, it remains to be determined how far the anti-apoptotic effects of infection extend laterally into the newly formed syncytium.

Monocytes are critical cells for protecting the body against infections through innate responses (phagocytosis, virus-induced release of soluble factors and cytokines). However, HCMV is a unique virus in that *in vivo* monocytes are a primary target (Rice *et al.* 1984; Dudding *et al.* 1989; Fish *et al.* 1995) and appear to be the site of latency and persistence (Kondo *et al.* 1994; Fish *et al.* 1996). Furthermore, monocytes have been hypothesized to be responsible for disseminating the virus throughout the body (Taylor-Wiedeman *et al.* 1994; Fish *et al.* 1995), and play a role in the manifestation of many HCMV-associated diseases (Dudding *et al.* 1989; Gura 1998; Murayama *et al.* 1998). While, undoubtedly, monocyte innate responses reduce peripheral blood virus levels, the present observations suggest that monocytes also play an important role in initiating infection-induced placental villitis.

HCMV rapidly upregulates ICAM-1 and induces resistance to apoptosis in infected cells (Skaletskaya *et al.* 2001; Chan *et al.* 2002; Arnoult *et al.* 2004). However, we also have seen an induction of TNFα secretion within 24 hrs of infection which induces trophoblast apoptosis in neighbouring non-infected cells (Chan *et al.* 2002). In this paper, we see that adhering monocytes increase apoptosis in the underlying infected trophoblast. It is unlikely monocytes would stimulate death in infected trophoblast since monocyte-induced apoptosis appears to be dependent on TNFα and HCMV infected cells are resistant to TNFα-induced death (Zhu *et al.* 1995; Chan *et al.* 2002). Our data (Fig 5.6) indeed shows trophoblast infected with gfp-HCMV were never TUNEL-positive. However, we have previously shown that HCMV is not only able to directly induce ICAM-1 expression but also via the release of TNFα and IL1β (Chan *et al.* 2004). *In vivo*

these cytokines could stimulate ICAM-1 expression at sites distant from viral infection. Monocytes could then adhere to these regions to induce local apoptosis.

The above observations suggest that HCMV can induce villitis by multiple mechanisms. We have previously shown HCMV to induce trophoblast death by inducing apoptosis in neighbouring non-infected cells via the release of TNFα (Chan *et al.* 2002). In this communication, we suggest that villitis induced by HCMV can be further enhanced by the accumulation of monocytes at sites of infection and that it too is mediated by TNFα. In the first example, TNFα diffusion would be apical (diffusing over the ST) or basal (diffusing into the CT or stroma). However, monocytes adhering to the ST would release TNFα over a broader area. The two mechanisms could operate collaboratively in initiating placental villitis.

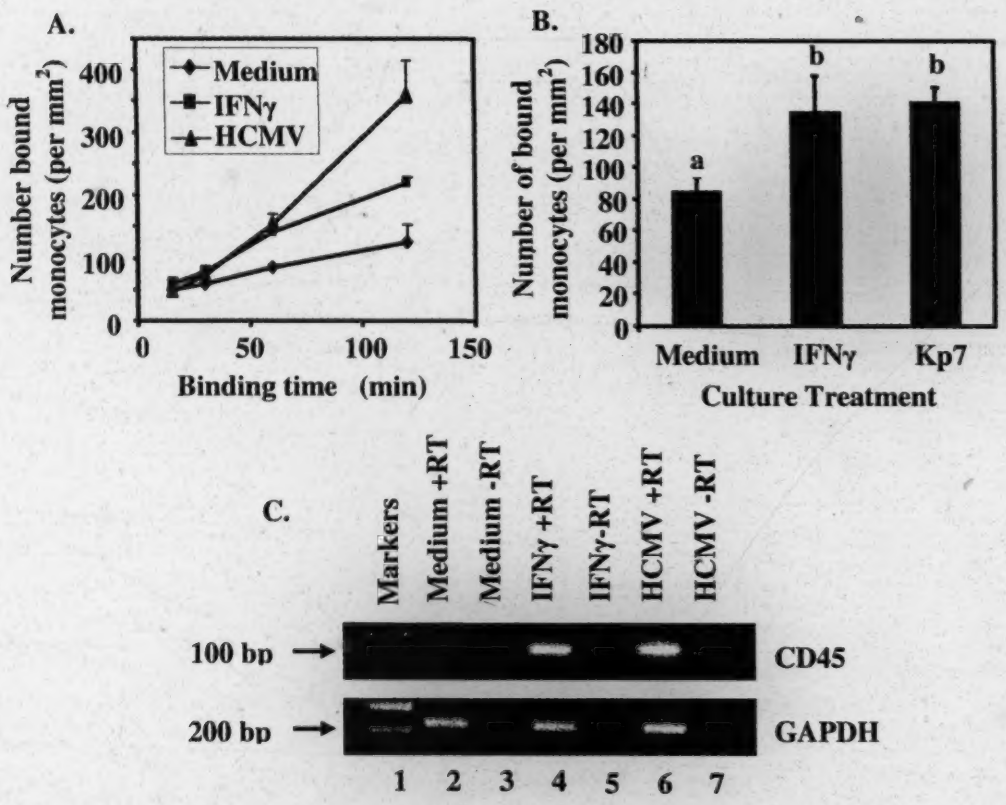

Figure 6.1: Adhesion of monocytes to ST cultures infected with HCMV.

ST cultures were treated with medium alone (control), IFNγ (100 U/ml) or challenged with HCMV strain AD169 (**A, B**) or Kp7 (**C**) at a MOI of 10 for 24 hrs. (**A**) At time points indicated, the number of bound monocytes (CD45-positive) per mm^2 was determined by immunofluorescence analysis as described in Materials and Methods. (**B**) Following 2 hrs adhesion period the number of bound monocytes per mm^2 was determined as described in Materials and Methods. Depicted is the mean $\pm$ SD of three independent experiments using cells from two different placentae and a fourth experiment using cells from another placenta showed the same trend. Experimental groups within each panel labeled with different letters (a or b) are significantly different ($P<0.05$). (**C**) RT-PCR of CD45 and GAPDH expression in ST /monocyte co-culture. RNA was isolated following a 2 hr monocyte adhesion period. Lane 1 = ladder; lanes 2, 4, 6 = treatments plus reverse transcriptase (+RT); lanes 3, 5, 7 = treatments minus reverse transcriptase (-RT). This gel is representative of four independent experiments using trophoblast isolated from three different placentae.

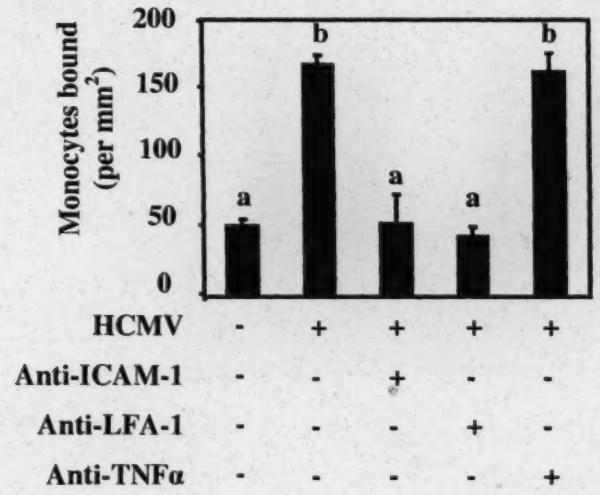

Figure 6.2: The effect of antibody against ICAM-1 and LFA-1 on monocyte binding to ST cultures.

AD169 infected ST cultures or monocytes were preincubated for 1 hr in the presence of 10 μg/ml anti-ICAM-1, anti-LFA-1 or anti-TNFα antibody, respectively, prior to addition of monocytes to all ST cultures. Following 2 hrs of co-culture, the number of monocytes bound per mm^2 was determined. Depicted is the mean ± SD of pooled results from three independent experiments using cells from two different placentae and a fourth experiment with another placental preparation showed the same trends. Experimental groups within each panel labeled with different letters (a or b) are significantly different ($P<0.05$).

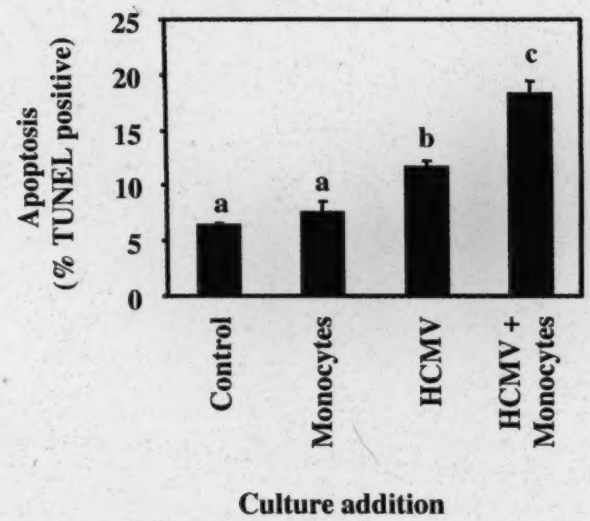

Figure 6.3: Monocyte-induced apoptosis of HCMV-infected ST cells.

HCMV-infected or non-infected ST cultures were incubated with or without monocytes for 24 hrs and the fraction of trophoblast apoptotic nuclei were determined by TUNEL analysis. Depicted is the mean $\pm$ SD of pooled results from three independent experiments using cells from two different placentae. Two other experiments with cells from yet another placenta showed the same trends. Experimental groups within each panel labeled with different letters (a, b or c) are significantly different ($P<0.05$).

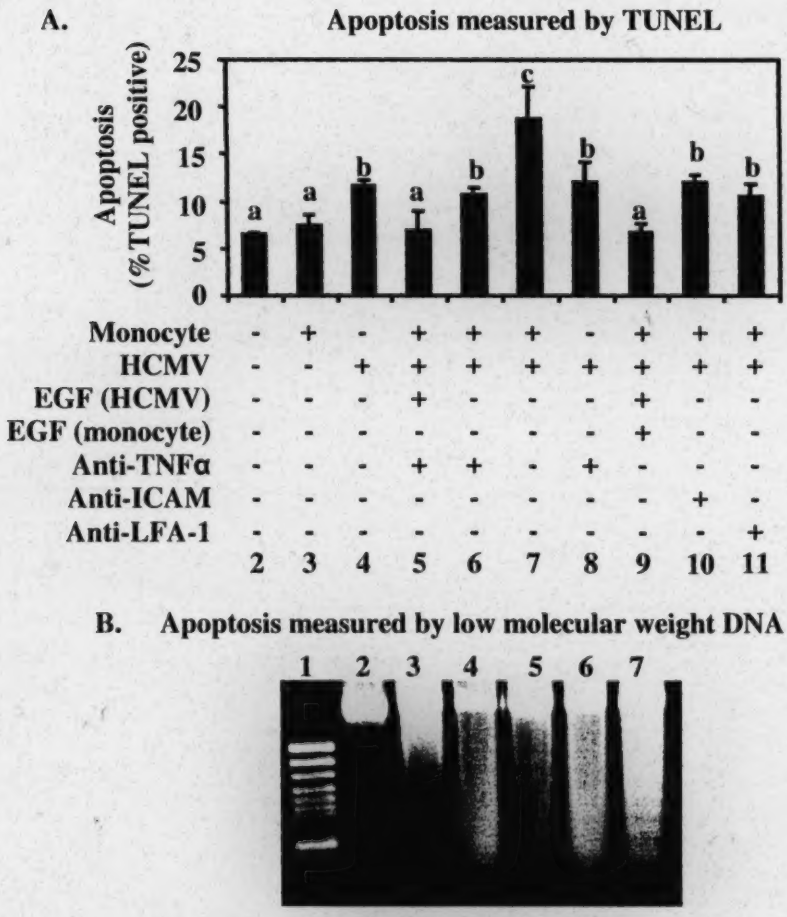
A.
Apoptosis measured by TUNEL
Apoptosis (%TUNEL positive)
25
20
15
10
5
0
a
a
b
a
b
c
b
a
b
b
Monocyte - + - + + + - + + +
HCMV - - + + + + + + + +
EGF (HCMV) - - - + - - - + - -
EGF (monocyte) - - - - - - - + - -
Anti-TNFα - - - + + - + - - -
Anti-ICAM - - - - - - - - + -
Anti-LFA-1 - - - - - - - - - +
2 3 4 5 6 7 8 9 10 11
B. Apoptosis measured by low molecular weight DNA
1 2 3 4 5 6 7

Figure 6.4: The effect of antibodies against TNFα, ICAM-1 and LFA-1 on monocyte-induced trophoblast apoptosis.

HCMV infection of ST cultures was carried out in the presence [EGF(HCMV)] or absence of EGF. Following infection cultures were preincubated for 1 hr in the presence of 10 μg/ml anti-ICAM-1, 10 μg/ml anti-TNFα antibodies or EGF [EGF(monocyte)]. Monocytes were preincubated with anti-LFA-1 antibody prior to being added to ST cultures. **(A)** The percent TUNEL-positive nuclei were determined after 24 hrs of culture, as described in Materials and Methods. Depicted is the mean ± SD of pooled results from three independent experiments using cells from two different placentae and a fourth experiment with cells from yet another placenta showed the same trends. Experimental groups within each panel labeled with different letters (a, b or c) are significantly different ($P<0.05$). **(B)** Cells were lysed and the DNA isolated and analyzed by 1.5% agarose electrophoresis as described in Materials and Methods. Molecular weight markers were run in lane 1. Lanes 2-7 correspond to treatments indicated in panel A. This experiment was repeated three times using trophoblast from three different placentae with similar results.

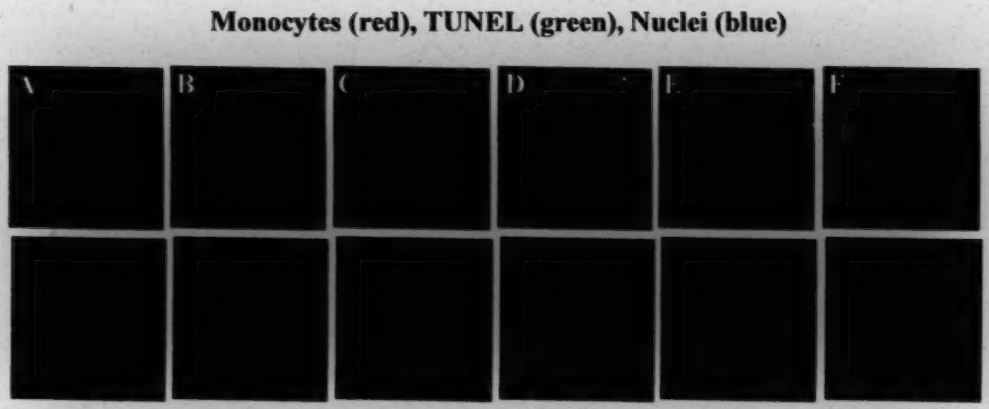

Figure 6.5: Immunofluorescent analysis of trophoblast apoptosis and adherent monocytes.

Photomicrographs of: **A)** ST cultures, **B)** ST cultures co-incubated with adhering monocytes, **C)** ST cultures infected with HCMV **D)** ST cultures infected with HCMV and co-incubated with adhering monocytes and **E)** ST cultures treated with EGF during HCMV infection and monocyte co-culture **F)** ST cultures treated with EGF during HCMV infection and pre-treated with anti-TNFα (10 μg/ml) prior to the addition of monocytes. Following 24 incubation, cultures were stained for monocytes (CD45-positive, red) and apoptosis (TUNEL-positive, green). Total nuclei were visualized using DAPI staining and are shown in lower panel.

Figure 6.6: Photomicroscopic analysis of gfp-HCMV infection, monocyte binding and apoptosis in ST cultures.

Photomicrographs of the same ST culture infected with (**A**) gfp-HCMV (green) and (**B**) stained for monocytes (CD45-positive, red) and (**C**) apoptosis (TUNEL-positive, blue). (**D**) Overlay of all three images. Cultures were carried out as described in the Methods and Materials.

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CHAPTER 7: DISCUSSION

Despite congenital HCMV infection being a major public health issue costing billions annually to support children with HCMV disease, little is known about the mechanisms of vertical transmission (Britt and Alford 1996). Immunohistochemical analysis of sections from term placentae displaying chronic villitis revealed IE (Muhlemann *et al.* 1992; Sinzger *et al.* 1993) but not E (Muhlemann *et al.* 1992) or L (p150) (Sinzger *et al.* 1993) antigens in the villous trophoblast, suggesting abortive infections (Sinzger *et al.* 1993). Tissue sections of villous explants challenged with HCMV for 4 days express IE proteins in the CT and extremely rarely in the ST (Fisher *et al.* 2000). Villi that were infected with HCMV *in utero* show a similar pattern of infection where the ST were often spared and the CT express a number of viral proteins. Non-permissive infection of ST suggested a model by which vertical transmission occurs through the anchoring villi. Infected microvascular endothelial cells have been shown to transmit HCMV to invasive CT (Maidji *et al.* 2002), indicating that infection could spread from the maternal uterine cells to the extravillous/invasive CTs, thus bypassing the ST. Indeed, any infection of the ST layer would be rare and likely to occur later in the infection process (Fisher *et al.* 2000).

Although the aforementioned studies indicate non-productive infection in the villous placenta, *in situ* hybridization revealed HCMV DNA in stromal cells and in the trophoblast of term placentae with chronic villitis (Sachdev *et al.* 1990; Kumazaki *et al.* 2002). Placentae from first or second trimester abortions contain nuclear inclusions frequently in stromal cells (Schwartz *et al.* 1992) and more rarely in trophoblasts (Garcia *et al.* 1989) with expression of early antigen pp65 in the trophoblast (van Lijnschoten *et al.* 1994; Halwachs-Baumann *et al.* 1998) suggesting a permissive trophoblast infection. Explants of first trimester placentas demonstrated complete replication of HCMV in villous trophoblast cells (Gabrielli *et al.* 2001) and *in vitro* cultured term villous trophoblasts can be productively infected in culture (Halwachs-Baumann *et al.* 1998; Hemmings *et al.* 1998). However, there does not appear to be any basal release of HCMV progeny particles from polarized cultured villous trophoblast (Hemmings and

Guilbert 2002) indicating that vertical transmission may not occur through the villous placenta even in the presence of a productive infection.

Alternatively, HCMV infection is a major cause of chronic villitis (inflammation) (Schwartz *et al.* 1992; Benirschke and Kaufmann 2000) and placentae from infants diagnosed with congenital HCMV infection often display villous destruction (Garcia *et al.* 1989; Greco *et al.* 1992). Microscopic examination revealed disruptions in the syncytiotrophoblast barrier through the formation of lesions (Benirschke *et al.* 1974; Garcia *et al.* 1989; Schwartz *et al.* 1992; Sinzger *et al.* 1993). This ability of HCMV to destroy trophoblast was demonstrated in placental explants (Amirhessami-Aghili *et al.* 1987). Therefore, I propose that the loss of trophoblast barrier function can allow for vertical transmission of HCMV.

In addition to vertical transmission, trophoblast loss could potentially impair villous placental function decreasing oxygen and nutrient delivery to the fetus. Elevated levels of apoptosis in the villous trophoblast have been demonstrated in IUGR pregnancies (Smith *et al.* 1997; Erel *et al.* 2001; Ishihara *et al.* 2002) suggesting apoptosis to be involved in the pathophysiologic mechanism. Primary maternal HCMV infection acquired later in pregnancy leads to 25% of neonates exhibiting IUGR (Istas *et al.* 1995). I propose that HCMV-induced villous trophoblast loss leads to placental insufficiency associated with IUGR.

In these studies I have investigated multiple mechanisms by which HCMV infection of culture villous trophoblast stimulates apoptosis possibly leading to vertical transmission and placental dysfunction.

HCMV Infection Indirectly Leads to Trophoblast Apoptosis (Fig 7.1)

The accumulation of monocytes is a hallmark of chronic inflammation. Indeed, the major infiltrating leukocytes in villitis associated with HCMV or with villitis of unknown etiology are mononuclear phagocytes (Altemani 1992; Greco *et al.* 1992), which appear to cluster at sites of trophoblast loss (Garcia *et al.* 1989; Greco *et al.* 1992). Monocyte adhesion to ST cultures *in vitro* is mediated by ICAM-1 and can be upregulated by inflammatory cytokines IL1, TNFα and IFNγ (Xiao *et al.* 1997). Monocytes binding to IFNγ-activated ST cultures induce trophoblast damage in a TNFα-

dependent manner (Garcia-Lloret *et al.* 2000) suggesting that villous ST upregulation of ICAM-1 is an early step in villitis.

HCMV infection results in increased ICAM-1 expression in a number of different endothelial and epithelial cell lines (Grundy and Downes 1993; Craigen and Grundy 1996; Watanabe *et al.* 1996; Shahgasempour *et al.* 1997; Burns *et al.* 1999; Dengler *et al.* 2000); however, nothing is known of the effect of HCMV on the expression of ICAM-1 of villous trophoblasts. In Chapter 5, I show that HCMV infection of ST cultures can induce surface ICAM-1 expression by a number of mechanisms. HCMV stimulates the secretion of inflammatory cytokines IL1β and TNFα to induce ICAM-1. The presence of neutralizing antibodies to both cytokines during infection significantly reduced surface ICAM-1 but did not completely block induction. Further detailed examination revealed that neutralizing antibodies did not block HCMV ICAM-1 induction on infected cells suggesting direct upregulation of ICAM-1. Indeed, others have found HCMV to directly induce ICAM-1 expression on HEL and HUVEC cells (Craigen and Grundy 1996; Burns *et al.* 1999; Kronschnabl and Stamminger 2003). In HUVECS, HCMV IE proteins acted on the ICAM-1 gene promoter to stimulate transcription (Burns *et al.* 1999; Kronschnabl and Stamminger 2003). My results indicate HCMV IE proteins can also induce trophoblast ICAM-1 but more studies are needed to determine whether IE proteins are directly stimulating ICAM-1 or leading to the secretion of cytokines such as IL1β and TNFα.

Monocyte binding to the ST is mediated by ICAM-1/LFA-1 interactions and I show that HCMV infected cultures bind and increase the number of monocytes. The increase in binding capacity appears to be solely due to the upregulation of ICAM-1 since neutralizing antibodies reduced monocyte adhesion to similar levels seen in the uninfected control. Co-culture of monocytes and infected ST resulted in higher levels of apoptosis than in infected ST alone. TNFα, which plays a central role in the development of chronic inflammation, is secreted by monocytes (Goldsby *et al.* 2003). Similar to others, I found that the presence of neutralizing TNFα antibody did not inhibit monocyte adhesion but was able to block monocyte-induced ST apoptosis (Garcia-Lloret *et al.* 2000). These studies indicate that trophoblast loss associated with HCMV infection can be mediated by increased monocyte adhesion to ST.

HCMV Infection Directly Leads to Trophoblast Apoptosis (Fig 7.2)

HCMV-induced histopathologic lesions bearing HCMV antigens were consistently localized in the trophoblastic cells covering placental villi in villous explants (Amirhessami-Aghili *et al.* 1987). I found that HCMV induced apoptosis in cultured ST within 24 hours of infection. HCMV IE gene products can induce transcription factors necessary for TNFα promoter activity to stimulate protein production (Geist *et al.* 1994; Geist *et al.* 1997). Individual expression of IE1-72, IE2-55 and IE2-86 were able induce trophoblast death. Both HCMV and HCMV IE-induced apoptosis could be blocked using neutralizing TNFα antibody or EGF, a known inhibitor of TNFα-induced apoptosis (Garcia-Lloret *et al.* 1996). Interestingly, UV-inactivated HCMV could also induce a significant amount of apoptosis which could be blocked by TNFα antibody. UV-inactivated virions appear to activate the TLR2 signaling cascade stimulating TNFα gene transcription. Treatment with CHX did not block the induction of TNFα transcription indicating upregulation is independent of de novo protein synthesis. Neutralizing antibody to TLR2 did not affect binding of HCMV viral particles to the ST surface but completely abrogated induction of TNFα by UV-inactivated HCMV and consequently induction of trophoblast apoptosis. Taken together, these results suggest that early events during the HCMV replication cycle can stimulate trophoblast loss associated with HCMV-induce villitis.

Role of HCMV IE Proteins in HCMV-Induced Trophoblast Apoptosis

From the studies presented, it is apparent that the major HCMV IE proteins, IE1-72, IE2-55 and IE2-86, play a role in HCMV-induced villitis. Individual expression of IE proteins in trophoblast stimulated both apoptosis and ICAM-1 expression, albeit to varying degrees. Initially, it was somewhat surprising to find that all three proteins could stimulate the same events; however, it is clear that these three IE proteins share some similar structure and function.

These alternatively spliced IE mRNAs originate from the same IE region under the control of the MIE promoter. IE1-72 is the most abundant transcript and is composed of exons 1 through 4. The other two major transcripts, IE2-55 and IE2-86 mRNAs, have

the same first three exons as in the IE1-72 mRNA but contain exon 5 in place of exon 4. IE2-55 is identical the IE2-86 except that an alternative splicing event within exon 5 results in a 154 aa deletion. Since all three proteins share the first three exons, the first 85 aa in the N-terminal sequence are identical. This structural similarity may account for some of the analogous functions while the remaining differing sequences likely account for the divergent activities of each protein.

All three IE proteins are able to upregulate expression of NF-κB by transactivation of the p65 promoter while IE2-55 can also stimulate NF-κB via the p105/p50 promoter (Yurochko *et al.* 1995). The IE proteins physically interact and cooperate with Sp1 to increase promoter transctivation (Yurochko *et al.* 1997). This induction of NF-κB by the IE1 and IE2 proteins likely explains the protection from TNFα-induced apoptosis conveyed to trophoblast (Chapter 3) and Hela cells (Zhu *et al.* 1995). However, the stimulation of NF-κB likely also accounts for the induction of apoptosis seen in neighbouring non-infected trophoblast (Chapter 3). IE1-72 has been shown to upregulate transcription factors, including NF-κB, necessary for the transcription of TNFα (Geist *et al.* 1997). In fact, both IE1 and IE2 gene products can increase TNFα promoter activity, steady state mRNA, and protein production (Geist *et al.* 1994).

Similar to others, I have found that the IE1 and IE2 proteins stimulate ICAM-1 (Chapter 5) (Burns *et al.* 1999; Kronschnabl and Stamminger 2003). Contrary to these studies where upregulation was a direct consequence of IE genes acting on the ICAM-1 promoter, the relatively low transfection rates (~10%) and the widespread increase in ST surface ICAM-1 expression suggest a soluble factor is being released into the culture media inducing ICAM-1. Induction of cytokines such as TNFα and IL-1β by IE1 and IE2 gene products can promote surface trophoblast ICAM-1 expression (Geist *et al.* 1994; Geist *et al.* 1997; Wara-aswapati *et al.* 1999; Yang *et al.* 2002; Wara-Aswapati *et al.* 2003). However, since HCMV infection can also directly stimulate ICAM-1 (Chapter 5), it is likely that IE1 and IE2 proteins also directly upregulate ICAM-1 expression. Further investigation will be needed to determine the mechanism of ST ICAM-1 induction by IE gene products.

Role of NF-κB During HCMV-Induced Paracrine Trophoblast Apoptosis (Fig 7.3)

NF-κB appears to play a central role in both HCMV viral replication and paracrine induced trophoblast apoptosis. Engagement of gB with TLR2 results in the activation of NF-κB (Compton *et al.* 2003; Rassa and Ross 2003). Translocation of NF-κB to the nucleus can stimulate transcription from promoters with NF-κB binding sites including those controlling the HCMV IE, TNFα and ICAM-1 genes (Drouet *et al.* 1991; Ledebur and Parks 1995; Prosch *et al.* 1995). Production of HCMV IE proteins, which are essential for viral replication and dependent on NF-κB activity (DeMeritt *et al.* 2004), can further induce transcription of both NF-κB subunits (Yurochko *et al.* 1995), TNFα (Geist *et al.* 1994; Geist *et al.* 1997) and ICAM-1 (Kronschnabl and Stamminger 2003). Upregulation of ICAM-1 allows for binding of monocytes via LFA-1. Secretion of TNFα by infected trophoblasts or bound monocytes can have autocrine effect leading to activation of NF-κB promoting viral replication (Prosch *et al.* 1995) or paracrine effect leading to apoptosis of neighboring non-infected cells (Chapter 3, 4 and 6).

Model for Vertical Transmission of HCMV

These studies address major issues associated with vertical transmission across the villous placenta. IE expression has been observed in trophoblast of sections from terms placentae infected with HCMV (Muhlemann *et al.* 1992; Sinzger *et al.* 1993) but not E (Muhlemann *et al.* 1992) or L (p150) (Sinzger *et al.* 1993) antigens, indicating non-permissive infection (Sinzger *et al.* 1993). *In vitro*, ST cultures support productive HCMV infection, albeit at low levels (Hemmings *et al.* 1998). Infected polarized villous ST cultures release approximately 5% of progeny virus from only the apical surface (i.e. towards the maternal circulation) (Hemmings and Guilbert 2002). These observations do not support an infection and release model of vertical transmission. I suggest a model of vertical transmission where productive infection of the villous ST may not necessarily be required.

During a HCMV infection, monocytes are the primary cell type infected in blood (Taylor-Wiedeman *et al.* 1991; Sinclair and Sissons 1996; Sinzger and Jahn 1996) and are the predominant infiltrating cell found in infected organs (Booss *et al.* 1989). Although initial infection of monocytes by HCMV is not productive, infection can lead

cellular changes to promote differentiation into macrophages and thus permissiveness for viral replication (Smith *et al.* 2004). There are twice as many monocytes per unit volume of blood in the intervillous space as in peripheral blood (Moore *et al.* 2003) suggesting a low affinity interaction between monocytes and the villous ST. This interaction could allow infected monocytes to adhere, mature and occasionally transfer an infection to a susceptible region of the ST.

Binding of the progeny particle to TLR2 on the ST surface induces secretion of TNFα inducing apoptosis to both ST and CT (Chapter 4). Subsequent expression of IE genes would further simulate TNFα production and trophoblast apoptosis in non-infected regions of the ST (Chapter 3). Moreover, HCMV infection can directly stimulate trophoblast ICAM-1 expression in infected cells and indirectly in uninfected cells via secretion of TNFα and IL-1β (Chapter 5). Expression of the IE1 and IE2 proteins alone could also stimulate ICAM-1 suggesting that a fully productive infection is not required for upregulation. HCMV infected monocytes exhibit increased TNFα production (Geist *et al.* 1994); thus, increased binding of activated monocytes to non-infected regions of the ST would stimulate apoptosis in a TNFα-dependent manner (Chapter 6). Overall, these studies indicated early events in the viral life cycle would be sufficient to lead to villous trophoblast damage. Formation of breaks in the ST barrier could allow for vertical transmission of HCMV.

Implications for IUGR

Pregnancies complicated by IUGR exhibit increased levels of TNFα in the maternal serum (Bartha *et al.* 2003) and amniotic fluid (Stallmach *et al.* 1995) which is likely due to increased placental TNFα production (Holcberg *et al.* 2001). Cultured villous trophoblasts and villous explants from IUGR pregnancies not only exhibit increased TNFα production but also increased sensitivity to TNFα (Crocker *et al.* 2003; Crocker *et al.* 2004). Indeed placentae isolated from IUGR births have a significantly higher frequency of trophoblast apoptosis (Smith *et al.* 1997; Ishihara *et al.* 2002). Taken together, these observations imply apoptosis to be involved in the pathophysiologic mechanisms of IUGR. Increased apoptosis could result in smaller placentas and hence growth restricted babies. Alternatively, it is also possible that the increased incidence of

apoptosis is a result of the pathologic processes leading to IUGR. Inadequate implantation can decrease oxygen delivery to the villous placenta and fetus thus accounting for IUGR. Further, hypoxia is known to induce trophoblast apoptosis (Levy *et al.* 2000). Increased placental apoptosis could be interpreted as an effect of poor placentation rather than as a cause of the disease state.

Whatever the reason for the increase in trophoblast apoptosis, undoubtedly there is a correlation between placenta weight and fetal weight. In both normal and IUGR pregnancies decreasing placenta size decreases fetal weight (Pardi *et al.* 2002). Examination of IUGR placentae show approximately 63% of cells undergoing apoptosis to be trophoblast (Smith *et al.* 1997; Erel *et al.* 2001), the major function of which is nutrient delivery from maternal to fetal circulation (Benirschke and Kaufmann 2000). The incidence of apoptosis in ST is 4% to 8.4% (Erel *et al.* 2001; Ishihara *et al.* 2002) verses <1% in uncomplicated pregnancies (Ishihara *et al.* 2002). An increase in apoptosis reflects increased trophoblast turnover and loss. Indeed, enhanced syncytial knot formation is characateristic of IUGR pregnancies (van der Veen and Fox 1983). Taking into account the 9 month gestation period and that the placenta is made up of 40% trophoblast, 47% stroma, and 13% endothelial (Mayhew *et al.* 1994), accelerated loss of trophoblast (as well as stroma and endothelial) would significantly decrease placental growth. Since trophoblast death directly impacts placental size, it is important to investigate factors that influence trophoblast apoptosis.

Primary HCMV infection acquired later during pregnancy results in approximately 25% of affected neonates displaying IUGR (Istas *et al.* 1995). Chronic villitis, a risk factor for IUGR in humans (Smith *et al.* 1997), is a clinical outcome of HCMV placental infection (Garcia *et al.* 1989; Schwartz *et al.* 1992; Sinzger *et al.* 1993; Benirschke and Kaufmann 2000). HCMV infection of extravillous CT downregulates metalloproteinase activity and invasiveness possibly leading to decreased placentation (Yamamoto-Tabata *et al.* 2004). Consequently, decreased oxygen delivery to the placenta could result in increased trophoblast apoptosis. Alternatively, I show that HCMV infection stimulates ST apoptosis in non-infected cells by inducing the secretion of TNFα from infected trophoblast via activation of the TLR2 pathway or by transactivation of the TNFα promoter by IE proteins. Furthermore, HCMV infection can

promote the binding of TNFα secreting monocytes. These studies indicate multiple mechanisms by which HCMV can induce higher placental trophoblast apoptosis.

Future Directions

Monocytes have been proposed to be the vector for viral disemmation (Rice *et al.* 1984; Taylor-Wiedeman *et al.* 1991; Fish *et al.* 1996; Smith *et al.* 2004). Although monocytes are non-permissive for viral replication, HCMV infection can promote monocyte-to-macrophage differentiation dependent on 3,4,5 triphosphate kinase (PI(3)K)-activity and independent of new viral gene expression. HCMV-differentiated macrophages are permissive for replication (Smith *et al.* 2004). Consequently, progeny virus can be transmitted to adjacent tissue. My studies have shown that HCMV infection and IE protein expression can stimulate the upregulation of ICAM-1 resulting in increased monocyte adhesion. The connumdrum is how does the ST initially become infected with HCMV. During a normal pregnancy, the intervillous space contains twice the number of monocytes when compared to peripheral blood (Moore *et al.* 2003) indicating a low affinity interaction between monocytes and the villous ST. During a primary HCMV infection, infected maternal monocytes could enter the villous space, adhere, mature and occasionally transfer an infection to a susceptible region of the ST. To address this possibility, gfp-HCMV infected monocytes could be co-cultured with uninfected ST for an extended period of time to allow for differentiation into macrophages. Expression of GFP in the underlying ST can be monitored for lateral transmission from the HCMV-differentiated macrophages.

During production of progeny, approximately equal amounts of HCMV and dense bodies (DBs) are produced (Sarov and Abady 1975). DBs consist mainly of viral glycoproteins and tegument proteins, with gB and pp65 as the major constituents, respectively (Sarov and Abady 1975; Irmiere and Gibson 1983; Severi *et al.* 1992). DBs have no nucleocapsid or viral DNA (Irmiere and Gibson 1983) but are able to efficiently infect cells and deliver their protein components. Further, DBs are able to induce both humoral and cellular immune responses (Pepperl *et al.* 2000); thus, DBs likely also contribute to the pathogenesis during HCMV infections. I have shown UV-treated replication deficient viral particles to induce apoptosis. These studies could be further

extended to DBs to determine whether these naturally occurring replication deficient viral particles can induce trophoblast damage.

HCMV glycoproteins have been shown to alter cellular gene expression which is likely mediated by the rapid dysregulation of cellular transcription factors (Boldogh *et al.* 1993; Kowalik *et al.* 1993). Soluble gB and anti-idiotypic antibody that mimics gH were able to induce Sp1 and NF-κB similarly to HCMV infection. Blocking gH and gB using specific antibodies inhibited rapid activation of Sp1 and NF-κB (Yurochko *et al.* 1997). Soluble gB induces secretion of inflammatory cytokines in a TLR2-dependent manner (Boehme and Compton 2004) and appears to directly interact with a heterodimer of TLR2 and TLR1 (Rassa and Ross 2003). I show that UV-inactivated HCMV can induce ST apoptosis by stimulating TNFα secretion in a TLR2-dependent manner. More detailed examination of the receptor-ligand interaction using purified HCMV glycoproteins to attempt to induce ST apoptosis would be an important next step. Purified recombinant gB has been proposed to be a potential vaccine to protect against congential HCMV infection (Bravo *et al.* 2003). Although such a vaccine is proven to be effective in preventing vertical transmission in guinea pigs models (Schleiss *et al.* 2003; Schleiss *et al.* 2004), if soluble gB induces trophoblast apoptosis it could be detrimental to the health of the placenta possibly resulting in IUGR neonates. These experiments would provide important insight in determining the potential risks to the fetus by using such a vaccine.

UV-inactivated HCMV induced apoptosis appears to be dependent on EGF induced trophoblast differentiation state. Inactivated viral particles were only able to stimulate apoptosis in ST cultures but not CT cultures. It would interesting to examine if ST cultures secrete additional molecules that aid in TNFα killing or if CT cultures express higher levels of anti-apoptotic molecules. In addition to EGF, a number of other factors including hCG and cAMP stimulate CT-to-ST differentiation *in vitro*. It would be interesting to determine if, by using these different differentiation factors, UV-inactivated HCMV induced trophoblast apoptosis was still dependent on differentiation state. Furthermore, villous explant tissue culture models, which more closely resemble the *in vivo* situation, could be used to determine if infection with HCMV or with UV-inactivated HCMV can still induce trophoblast apoptosis. Villous explants could be

cultured for 5 days to allow for ST formation (Black *et al.* 2004) after which could be challenged with the different viral particles and examined for trophoblast apoptosis.

Placental specimens from spontaneous macerated abortions showed a pronounced degree of dysmaturity of villous structures (Garcia *et al.* 1989). Villi were abundant but were variable size with irregualar contours and were covered by a thin ruptured layer of trophoblast. Placentae from liveborn infants whom were clinically normal at birth but developed neurological abnormalities within the first few months of life showed a higher degree of differentiation of villous structures, although dysmaturity was still present. HCMV binds and activates EGFR suggesting that infection may stimulate trophoblast differentiation. However, HCMV infection decreases secretion hCG, a differentiation marker associated with ST formation (Fig 7.3). Examining how HCMV affects trophoblast differentiation may give further insight to the pathogenicity associated with placental infection.

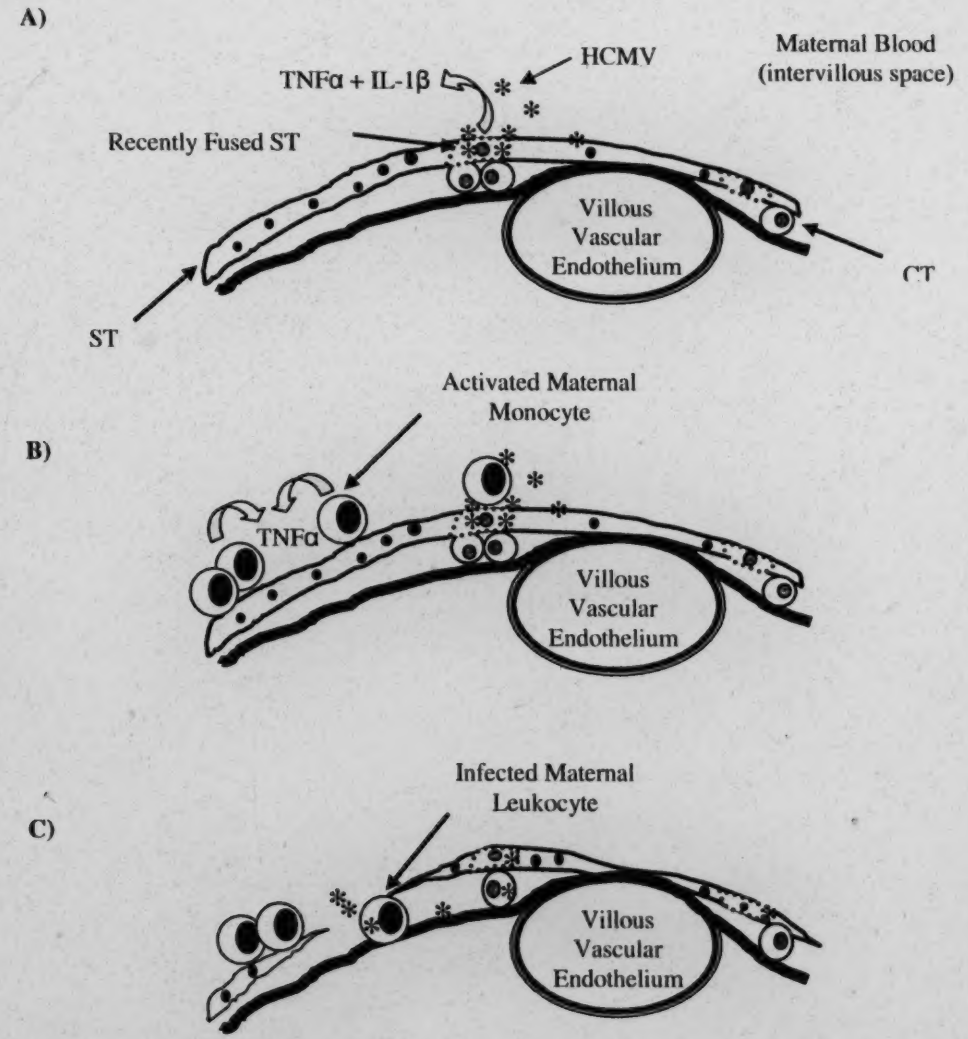

Figure 7.1: Hypothetical model of indirect damage induced by HCMV infection to the villous ST.

A) Viral replication and expression of HCMV IE proteins promote secretion of inflammatory cytokine TNFα and IL-1β which stimulate surface ICAM-1 expression. **B)** Activated maternal monocytes adhere to the ST surface via ICAM-1/LFA-1 interactions. Adherent monocytes at sites distant to viral infection release TNFα inducing apoptosis to the underlying trophoblast layer. **C)** Loss of trophoblast results in the formation of breaks in the ST barrier through which infected maternal leukocytes or cell-free virus may enter the villous stroma.

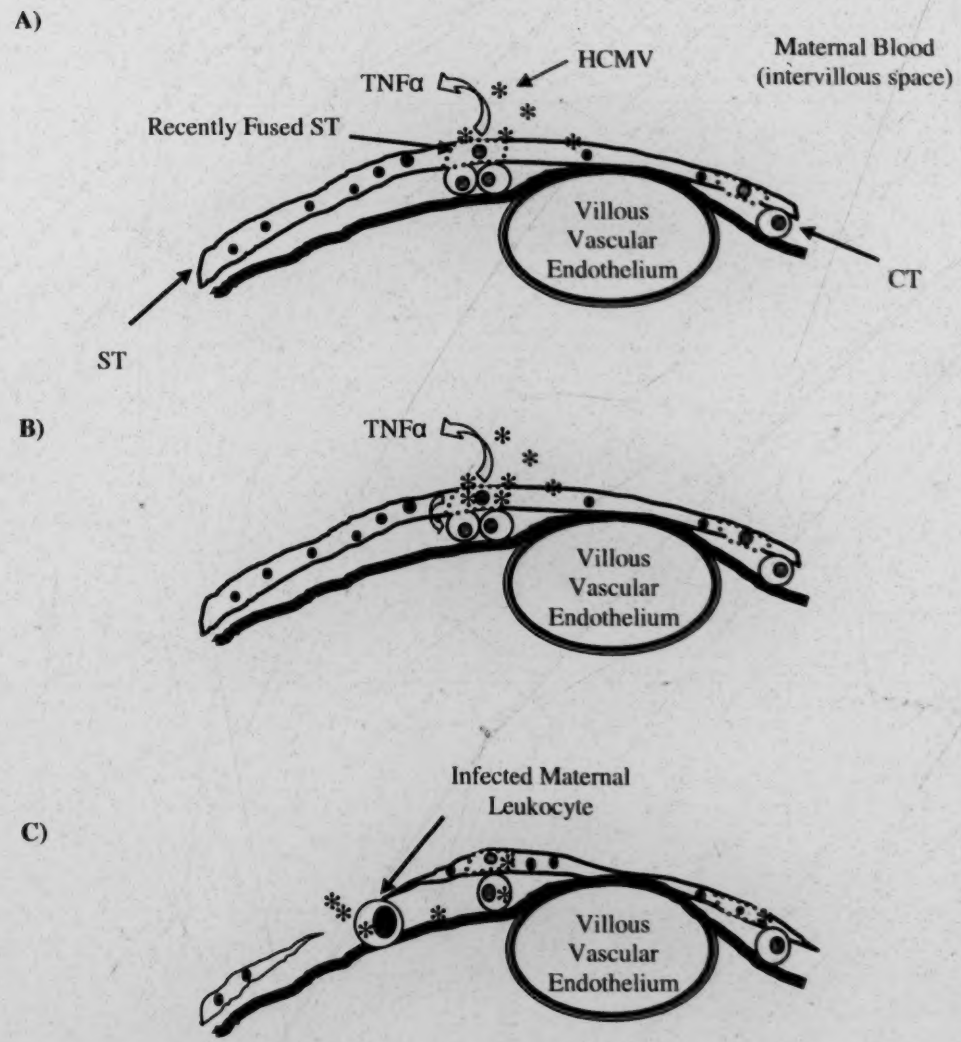

Figure 7.2: Hypothetical model of direct damage induced by HCMV infection to the villous ST.

A) HCMV adheres to the surface of the ST. Viral particles bind and activate TLR2 leading to the secretion of TNFα independent of viral protein expression. **B)** Internalization of HCMV and subsequent production of IE proteins stimulate further production of TNFα. **C)** TNFα can induce ST apoptosis at sites distant to viral infection. Loss of trophoblast results in the formation of lesions in the ST barrier through which infected maternal leukocytes or cell-free virus may enter the villous stroma.

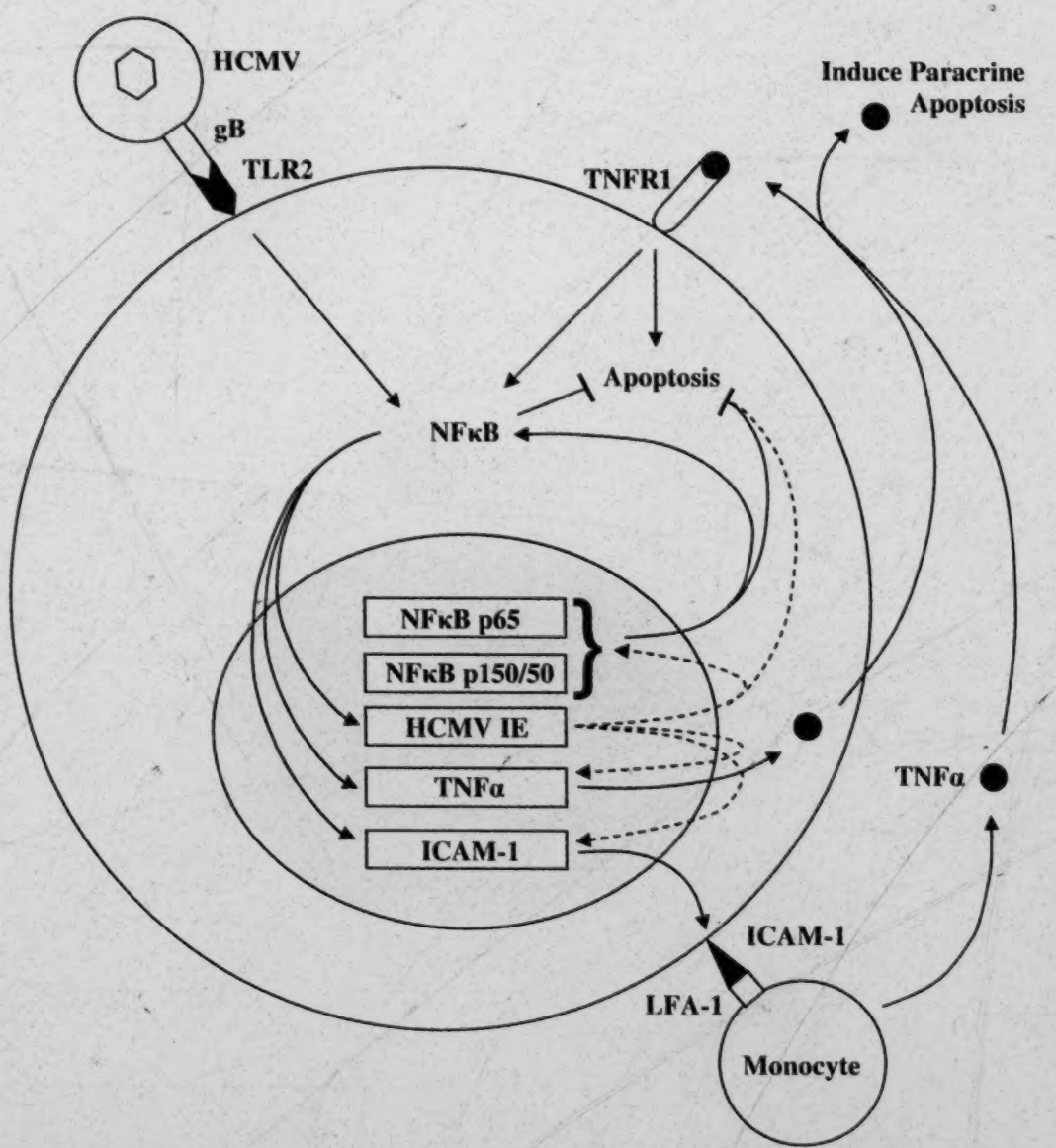

Figure 7.3: Hypothetical role of NF-κB in HCMV-induced paracrine trophoblast apoptosis.

Activation of NF-κB by infection with HCMV sets off a sequence of events leading to the secretion of TNFα. TNFα can then further stimulate NF-κB to promote viral replication in infected cells while inducing apoptosis in neighboring non-infected cells. See discussion for further detail.

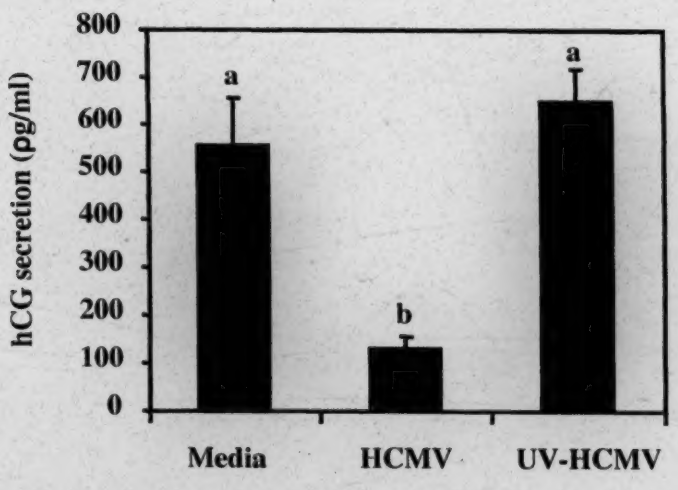

Figure 7.4: HCMV infection inhibits CT hCG secretion.

CT were plated in 96-well dish and cultured for 24 hrs. After incubation, cells were infected with AD169 or UV-inactivated virus at a MOI of 10. CT were then cultured for 5 days during which media was changed on days 1 and 3. Day 5 supernatant was collected and assessed for hCG secretion using a colourimetric assay system (DRG Diagnostics; Marburg Germany). The results shown are means $\pm$ SD of three independent experiments carried out with cells isolated by two placentae. Bars labeled with different letters (a or b) are significantly different ($P<0.05$).

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